ANTIARTHRITIC POLYHERBAL FORMULATION: DEVELOPMENT, STANDARDISATION AND EVALUATION

A dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
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In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY
IN
PHARMACOGNOSY

Submitted by

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Under the guidance of

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CERTIFICATE

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1. INTRODUCTION

Rheumatoid arthritis is an autoimmune disease in which there is joint inflammation, synovial proliferation and destruction of articular cartilage[1]. It is a disabling and painful inflammatory condition, which can lead to substantial loss of mobility due to pain and joint destruction.

Inflammation is a bodily response to injury, infection or destruction characterized by heat, redness, pain, swelling and disturbed physiological functions. Inflammation is normal protective response to tissue injury caused by trauma, noxious chemical or microbial agent. It is body response to inactivate or destroy the invading organisms, to remove the irritant and set the stage for the tissue repair. It is triggered by the release of chemical mediators from injured tissue and migrated cells[2].

It is a common disease having peak incidence in 3rd to 4th decades of life with 3-5 higher preponderance in female[3].

The most frequently affected joints are those of fingers and toes, wrists, knees, and ankles. Extra articular manifestations may occur and are frequently present in patients with severe disease which include ocular, pulmonary, hematologic, vascular, cardiac, neurologic and mucosal tissue.

The drugs commonly in use for the treatment of Rheumatoid arthritis include,

- Glucocorticoide (e.g Cortisone and prednisone)
- Non-steroidal anti-inflammatory drugs (NSAIDs e.g ibuprofen and Naproxone etc.,)
- Biological response modifier (e.g Tumour necrosis factor- alpha blocking agents).
- Disease-modifying anti rheumatic drugs (DMARDSs: e.g Methotrexate (MTX) and Leflunomide)

Herbal medicine, sometimes referred to as Herbalism or Botanical Medicine, is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history for their therapeutic or medicinal value. An herb is a plant or plant part valued for its medicinal, aromatic or savoury qualities. Herbal plants contain therapeutically active chemical substances that act upon the body[4]

Herbal medicines widely used in health-care in both developed and developing countries are complex chemical mixtures prepared from plants and are limited in their effectiveness because they are poorly absorbed when taken orally.

Herbal formulations have reached widespread acceptability as therapeutic agents for diabetic, arthritics, liver diseases, cough remedies, memory enhancers and adoptogens.

As per WHO definition, there are three kinds of herbal medicines:

Plant material

Processed plant material

Herbal products.

Herbal medicine products are dietary supplements that people take to improve their health and are sold as tablets, capsules, powders, teas, extracts and fresh or dried plants.

According to an estimate of the World Health Organization (WHO), about 80% of the world population still uses herbs and other traditional medicines for their primary health care needs[5]

Herbal medicine is a major component in all indigenous people traditional medicine and a common element in Ayurvedic, Homeopathic, Naturopathic. WHO notes that of 119 plant-derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value[6].

In the 20th century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples. Many drugs commonly used today are of herbal origin. About 25% of the prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material. Some are made from plant extracts; others are synthesized to mimic a natural plant compound. It was an integral part of the development of modern civilization[7].

Types of Herbal Medicine

Traditional Chinese Herbalism which is part of Traditional oriental Medicine, Ayurvedic Herbalism which is derived from Ayurvada and western Herbalism which is originally came from Greece and Rome to Europe and then spread to North and South America. Chinese and Ayurvedic Herbalism have developed into highly sophisticated systems of diagnosis and treatment over the centuries. Western Herbalism is today primarily a system of folk medicine[8]

Advantages of Herbal Medicine[9]

- Herbal medicine have long history of use and better patient tolerance as well as acceptance
- Medicinal plants have a renewable source, which is our only hope for sustainable supplies of cheaper medicines for the world growing population.
- Availability of medicinal plants is not a problem especially in developing countries like India having rich agro-climatic, cultural and ethnic biodiversity
- The cultivation and processing of medicinal herbs and herbal products is environmental friendly
- Cost-effectiveness-prescription drugs cost much more money than herbal medicines
- Lower side effects herbal medicines are generally a far heal their solution than prescription drugs due to potential harmful side effects caused by unpredicted body chemistry interactions

Disadvantage of Herbal Medicines

- Procedures for pure and genuine herbs are not available, so sub-standard and spurious herbs are there in the market
- Identification of exact mechanism and pharmacology of all herbal medicine is not available
- Adulteration ratio is very high
- Clinical and toxicological data was not available for all herbal medicine
- There is no much information available on the safety measures
- All herbal medicine are not tested with important parameters like microbial content, heavy metals content and pyrogens etc.

Herbal Medicine Today[10]

As the world modernized, so did herbal medicine. Laboratories produce synthetic medicines but there may be underlying side effects to the body. This is because they contain rich concentrations of chemical substances.

Chemists have analysed the components of herbs, then isolated and extracted the healing properties. The chemical moiety responsible for efficacy was synthesized in modern laboratories so they can be incorporated into to the modern medicines. Several herbal preparations are made in to pills, tablets and capsules, but still have the same benefits derived from natural herbs.

Herbal formulation[11]

An herbal 'formula' consists of a selective combination of individual herbal ingredients that are formulated for a specific ailment or group of disease-conditions. When herbs are combined together, they become more potent and effective within the body than single herb due to their activating or catalyzing influence upon one another. These combinations acts as powerful catalysts (with synergistic effects) in order to activate over own individual healing energies (or vital force) which permeate the entire organism and reside in each and every cell in our bodies.

The Capsule Dosage form[12]

The word "capsule" in the English language is derived from the Latin word "Capsula", which means a small box or container in more recent times, capsule has been used primarily to describe a solid oral dosage form, which consists of a container, usually made of gelatin filled with a medicinal substance. There are many forms of capsules and they can be divided into two main categories, which in current English usage are described by the adjectives "hard" and "soft". The 'hard capsule' consists of two separate parts, each semi-closed cylinder in shape. One part the 'cap', has a slightly larger diameter than the other, which is called the 'body' and is longer the cap fits closely over the body to form a sealed unit.

The soft gelatin capsule is also called as 'one piece'. Capsules are available in many size to provide dosing flexibility. Unpleasant drug tastes and odours can be masked by the tasteless gelatin capsules is one of the most frequently utilized dosage forms.

Hard gelatin capsules can be filled with a large varity of materials of different physicochemical properties (i.e dry solids, semi solid, non-aqueous liquids, etc) while soft gelatin capsule are generally used to contain liquid and semisolid materials.

Capsules offer many advantages

- Capsules, because of their elongated shape, are easy to swallow, which is one reason for the number of capsules- shaped tablets manufactured today.
- Flexibility of formulation is another advantage of the capsule dosage form. Howeverthe biggest formulation advantage of capsules is that there is less need for additional excipients.
- Since capsules are tasteless, they effectively mask any unpleasant taste or odour of their contents.
- They offer rapid release characteristics, due to the rapid dissolution rate of the capsules
- The use of hard capsules is also a common feature in clinical trials, as the filling of tablets or even capsules themselves will blind the dosage forms studied.
- The manufacture of capsules is much shorter process compared to that for other modern dosage forms (e.g Tablet).
- Controlled release can be achieved using capsules. Dry powder mixtures, granules, pellets and tablets can be filled into hard capsules. Moreover combination of two or three types (dry powder mixtures, tablets or pellets) also can be put into capsules.

Disadvantage of capsule

- The drugs which are hygroscopic absorb water from the capsule shell making it brittle and hence are not suitable for filling into capsules.
- The concentrated solutions which require previous dilution are unsuitable for capsules because if administered as such lead to irritation of stomach.

Standardization of Herbal Drugs[13]

"Standardization" of herbal drugs is not an easy task as numerous factors influence the bio efficacy and reproducible therapeutic effect. In order to obtain quality oriented herbal products, care should be taken right from the proper identification of plants, season and area of collection, their extraction, purification process and rationalizing the combination in case of polyherbal drugs. Standardization means adjusting the herbal drugs preparation to a define content of a constituent or a group of substances with known therapeutically activity respectively by adding excipients or by mixing herbal drugs or herbal drug preparation.

"Evaluation" of a drug means confirmation of its identity and determination of its quality and purity and detection of its nature of adulteration.[14]

Importance of standardization[15]

Quality control standards are very vital in developing the herbal formulations,

- To ensure batch to batch uniformity in contents.
- Confirmation of correct amount of dosage or extract per dosage unit.
- Positive control to indicate possible loss or degradation during manufacturing.

Two types of Standardization[16]

In the first category, "true" standardization, a definite phytochemical or group of constituents is known to have activity. Ginkgo with its 26% ginkgo flavones and 6% terpenes are the classic example. These products are highly concentrated and no longer represent the whole herb and are now considered as phytopharmaceuticals. In many cases they are vastly more effective than the whole herb.

The other type of standardization is based on manufacturers guaranteeing the presence of a certain percentage of marker compounds; these are not indicators of therapeutic activity or quality of the herb.

Quality Control of Herbal Medicine[17]

Quality can be defined as the status of a drug that is determined by identity, purity, content, and other chemical, physical and biological properties or by the manufacturing

processes. Quality control is a term that refers to processes involved in maintaining the validity of a manufactured product.

WHO Guidelines for Standardization of Herbal Formulation

- Quality control of crude drugs materials, plant preparations and finished products.
- Stability assessment and shelf life.
- Safety assessment; documentation of safety based on experience or toxicological studies.
- Assessment of efficacy by ethno medical information and biological activity evaluations.

2. REVIEW OF LITERATURE

Smilax zeylanica

Anita murali *et al* (2000) reported that antioxidant activity and HPTLC Studies on the root and rhizomes of *Smilax zeylanica.*[18]

SN Yoga narasimhan *et al* (2010) reported that pharmacognostical study of root and rhizomes of *Smilax zeylanica.*[19]

Prabhat kumar jena *et al* (2011) reported that phytochemical investigation and simultaneous study on antipyretic, anticonvulsant activity of leaves of *Smilax zeylanica.*[20]

Anita murali *et al* (2012) reported that hepato-protective activity of root and rhizomes of *Smilax zeylanica*.[21]

Sanjib saha *et al* (2013) reported that analgesic and antibacterial activity of leaves of *Smilax zeylanica* [22]

Lippia nodiflora

Ashokkumar *et al* (2001) reported that antioxidant activity of leaves of lippia nodiflora[23]

Asish tulshkar and Vijaya *et al* (2009) reported that neuropharmacological activity of leaves of lippia nodiflora[24]

Balamurugan *et al* (2011) reported that antidiabetic activity in leaves of lippia nodiflora[25]

Alireza ivanbakhsh *et al* (2012) reported that antimicrobial activity of leaves & flowers of *lippia nodiflora*[26].

Thomsan *et al* (2013) flavonoid fraction of hepatoprotective activity of leaves *lippia nodiflora*[27]

Asparagus racemosus

Hossain *et al* (2012) reported that cytotoxicity and in vitro antioxidant activity of *Asparagus racemosus* root extract.[28]

Ravishankar *et al* (2012) reported that preliminary phytochemical screening and in vitro antibacterial activity on *Asparagus racemosus* root.[29]

Januj joshi *et al* (2011) reported that anti stress activity of ethanolic extract of *Asparagus racemosus* willd root in mice.[30]

Javeed ahmed wani *et al* (2011) reported that phytochemical screening and aphrodisiac activity of *Asparagus racemosus* root.[31]

Ramachandran vadivelan *et al* (2011) reported that antioxidant and hypolipidemic activity of *Asparagus racemosus* on streptozotocin induced diabeticin rats.[32]

Bacopa monneri

R.K Goel *et al* (2000) reported that evaluation of in-vivo wound healing activity of *Bacopa monnieri* on different wound model in rats.[33]

Gopalakrishnan *et al* (2010) reported that potential effect of hepatoprotective activity of *Bacopa monnieri* on nitrobenzene induced liver damage in rats.[34]

Abhishek mathur *et al* (2010) reported that pharmacological investigation of *Bacopa monnieri* on the basis of antioxidant, antimicrobial & anti inflammatory activity.[35]

Sudha *et al* (2002) reported that anti convulsant activity of different extracts of *Bacopa monnieri* in animals.[36]

Aamir nazir *et al* (2001) reported that anti parkinsonian effects of *Bacopa monnieri* insights from transgenic and pharmacological caenorhabditis elegans models of parkinson's disease.[37]

Allium sativum

Meriga.B *et al* (2012) reported that insecticidal, antimicrobial and antioxidant activities of bulb extracts of allium sativum.[38]

Asaduzzaman *et al* (2010) reported that evaluation of antidiabetic, antihyperlipidemic and Hepatoprotective effects of alloxan induced diabetic rats.[39]

Ejaz *et al* (2003) reported that extract of Allium sativum in cancer chemoprevention.[40]

Venkatesh *et al* (2010) reported that identy of the immunomodulatory proteins from *Allium sativum* with the major garlic lectins or agglutinins.[41]

Belguith *et al* (2010) reported that study of the effect of aqueous garlic extract *Allium sativum* on some salmonella serovars isolates.[42]

Oldenlandia heynei

Abdur Rashid M *et al* (2010) reported that Hepatoprotective and antibacterial activity of ursolic acid extracted from *Oldenlandia heynei*[43]

Pandian S *et al* (2013) reported Hepatoprotective activity of methanolic extract of *Oldenlandia heynei* against D-galactosamine induced rat.[44]

Ahmad sazali hamzah *et al* (1998) Chemical constituent of *Oldenlandia heynei* Asean Review of Biodiversity and Environmental Concervation (ARBEC).[45]

Sigaravelu P, Shrishailappa B, Subban R. *in vitro* anti oxidant activity of *Oldenlandia heynei* www.ncbi.nlm.nih.gov (Bubmed).[46]

3. AIM AND OBJECTIVE

The aim of present study is to formulate an ayurvedic capsule for treatment of arthritis.

The formulated capsules are evaluated as per WHO guidelines and also performed pharmacological activity.

The objectives of the study were

1. Raw material analysis

- Studying the raw materials or ingredients of the formulation by carrying out the Preliminary raw materials analysis.

2. Preformulation development

- To formulate a capsule with six herbs by using preformulation studies.

3. Standardization of the best batches

- To standardise the physico-chemical parameters of the capsule.
- To analyse and Quantify of the presence of phytoconstituents in the capsule.
- To analyse the fingerprint using HPTLC for the polyherbal formulation.
- Establishing the safety pertaining to heavy metals, pesticide residue and microbial load analysis.

4. Stability studies

- Establishing stability of the formulation under accelerated condition of Temperature and humidity as per ICH guidelines.

5. Pharmacological evaluation of the formulation

- Acute toxicity studies
- Anti arthritic activity of the formulation using *in vitro* Parameters such as protein denaturation, Proteinase inhibitory activity and Membrane stabilization activity.
- *In vitro* antioxidant activity of the formulation such as reducing power assay, nitric oxide scavenging assay.
- Evaluation of the formulation for arthritic activity by adjuvant induced arthritis model in rats.

4. DISEASE PROFILE[47,48]

INTRODUCTION

Rheumatoid arthritis is a chronic inflammatory disease of unknown etiology marked by a symmetric, peripheral polyarthritis.

It is the most common form of chronic inflammatory arthritis and often results in joint damage and physical disability. Because it is a systemic disease, Rheumatoid arthritis may result in a variety of extra articular manifestations, including fatigue, subcutaneous nodules, lung involvement, pericarditis, peripheral neuropaty, vasculitis and hematological abnormalities.

CLINICAL FEATURES

The incidence of Rheumatoid arthritis increases between 25 and 55 years of age, after which it plateaus until the age of 75 and then decreases.

The presenting symptoms of Rheumatoid arthritis typically results from inflammation of the joints, tendons and bursae.

Patients often complain of early morning joint stiffness lasting more than 1 hour and easing with physical activity.

The earliest involved joints are typically the small joints of the hands and feet.

The initial pattern of joint involvement may be monoarticular, oligoarticular (≤ 4 joints), or polyarticular (> 5 joints), usually in a symmetric distribution.

EPIDEMIOLOGY

Rheumatoid arthritis affects approximately 0.5- 1% of the adult population world wide. Like many other autoimmune disease, Rheumatoid arthritis occurs more commonly in females than in males with a 3:1 ratio

Most of the theories center on the role of estrogens in enhancing the immune response. For example some experimental studies have shown that estrogen can stimulate production of tumor necrosis factor α (TNF α), a major cytokine in the pathogenesis of Rheumatoid arthritis.

ENVIRONMENTAL FACTOR

In addition to genetic predisposition, a host of environmental factors have been implicated in the pathogenesis of Rheumatoid arthritis.

The most reproducible of these environmental links is cigarette smoking.

HEMATOLOGY

A normochromic, normocytic anemia often develops in patients with rheumatoid arthritis and is the most common hematologic abnormality. The degree of anemia paralles the degree of inflammation, correlating with the levels of serum C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). Platlet counts may also be elevated in rheumatoid arthritis as an acute phase reactant. Immune mediated thrombocytopenia is rare in this disease.

PATHOLOGY

The pathologic hallmarks of RA are synovial inflammation and proliferation, focal bone erosions, and thinning of articular cartilage.

Chronic inflammation leads to synovial lining hyperplasia and the formation of pannus, a thickened cellular membrane of granulation- reactive fibrovascular tissue that invades the underlying cartilage and bone.

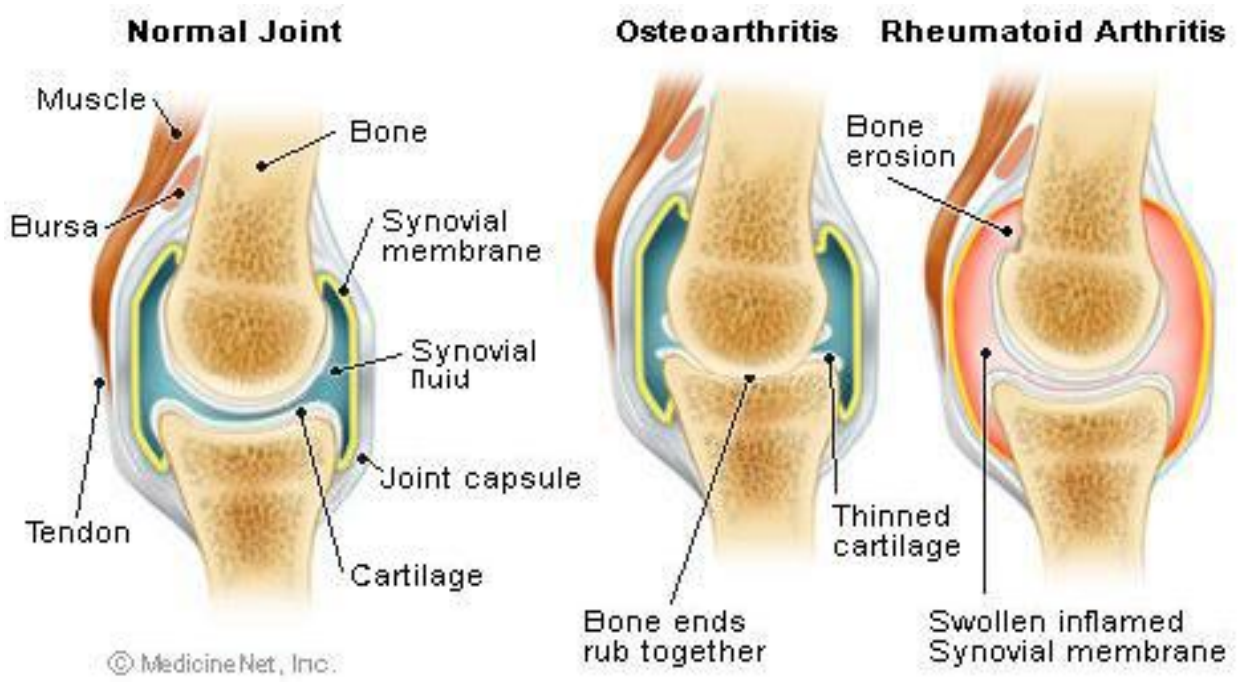


Fig 4.1 shows the pathological changes of rheumatoid arthritis joint in comparison with normal joint

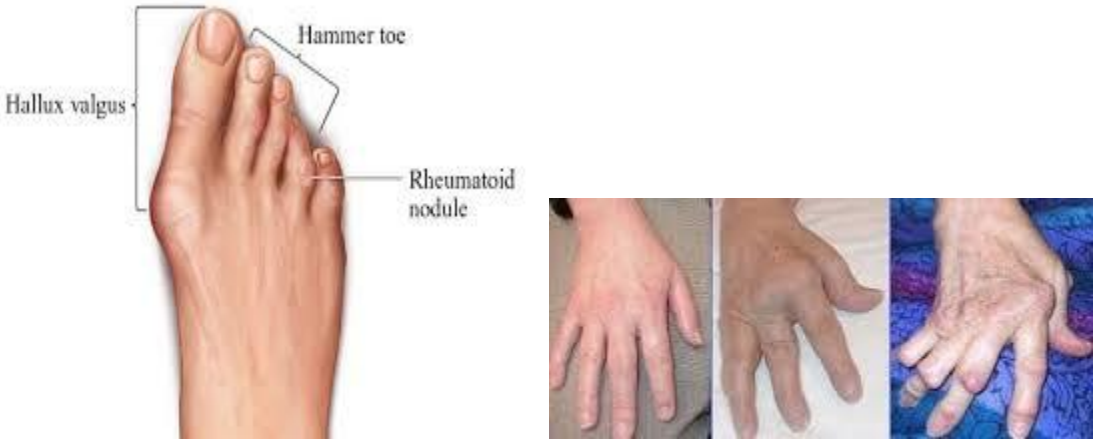


Fig 4.2 Rheumatoid arthritis of toes and fingers joint

MEDIATORS OF INFLAMMATION

Table 4.1Mediator of inflammation

Mediator	Principle sources	Actions
Cell derived		
Histamine	Mast cell, basophils, platelets	Vasodilation, vascular permeability endothelial activation.
Serotonin	platelets	Vasodilation,vascular permeability
Prostaglandins	Mast cell, leukocytes	Vasodilation, pain,fever
Leukotrienes	Mast cell, leukocytes	vascular permeability, chemotaxis, leukocyte adhesion and activation.
Platelet-activating factor	Mast cell, leukocytes	Vasodilation,vascular permeability leukocyte adhesion, chemotaxis, degranulation,oxidative burst.
Nitric oxide	Endothelium, macrophages	Vascular smooth muscle relaxation, killing of microbes
Cytokines (TNF, IL-1)	Endothelium, macrophages	Local endothelial activation, fever,/pain/anorexia/hypotenstion,shock
Chemokines	Leukocytes, activated macrophages	chemotaxis, leukocyte activation.
Plasma protein derived		
Kinins	Plasma (produced in liver)	Smooth muscle contraction, vasodilation, pain.
Protease activated during coagulation	Plasma (produced in liver)	endothelial activation,leukocyte recruitmant

DIAGNOSIS

The clinical diagnosis of Rheumatoid arthritis is largely based on sings and symptoms of a chronic inflammatory arthritis, with laboratory and radiographic results providing important supplemental information.

The new criteria include a positive test for serum anti cyclic citrullinated peptide antibodies as an item, which carries greater specificity for the diagnosis of Rheumatoid arthritis than a positive test for rheumatoid factor.

Table 4.2 Classification criteria for Rheumatoid factor

Classification	Rheumatoid Factors	Score
Joint involvement	One large joint (shoulder, elbow, hip, knee, ankle)	0
	2-10 large joints	1
	1-3 small joints(Thumb, Wrists)	2
	4-10 small joints	3
	>10 joints	5
Serology	Negative RF and negative ACPA	0
	Low (+) RF or low (+) anti CCP antibodies(≤3 times ULN)	2
	High positive RF or high (+) anti CCP antibodies (>3 times ULN)	3
Acute phase reactant	Normal CCP and normal ESR	0
	Abnormal CRP or normal ESR	1
Duration of symptoms	< 6 weeks	0
	≥6 weeks	1

LABORATORY FEATURES

- Synovial fluid analysis
- Joint imaging
  - Plain radiography
  - MRI
  - Ultrasound

TREATMENT

- Non Steroidal Anti- inflammatory Drugs
- Glucocorticoid
- DMARDS(Disease modifying antirheumatic drugs)
  - Hydroxyl chloroquine
  - Sulfasalazine
  - Methotrexate
  - Leflunomide
- Anti TNF α agent
  - Infliximab 3 mg/kg
  - Etanercept 50 mg
  - Golimumab 50 mg
  - Certolizumab 400 mg
  - Abatacept 500 mg
  - Anakinra 100 mg
  - Vituximab 1000 mg
  - Tocilizumab 4-8 mg/kg

5. PLANT PROFILE[49,50,51]

5.1 Smilax zeylanica

Synonym : *Smilax macrphylla Roxb.,*
Family : Smilacaceae

Part used : Root

Vernacular name

Hindi : Kumarika

Siddha/ Tamil : malattamari, kattu kodi

Sanskrit : vanamadhusnahi

Marathi : Kaitha

Telugu : Karivilanti

Kannada : Kodadantena

Macroscopy

Plants will grow as shrubs, A large climber; stems smooth, steriate, armed with a few small distant prickles or almost unarmed.

Phytochemical constituent:

Steroids, Terpenoids, Alkaloids, Flavones, Tannins, Phenolics and Saponins.

Therapeutic uses:

veneral disease, rheumatic swellings, given in urinary complaints and dysentery.

Dose: 50-100 mg

5.2 Asparagus racemosus

Synonym	: *Protasparagus racemosus (willd.)*
Family	: Liliaceae
Part used	: Root

Vernacular name

Hindi	: Marchuba
Siddha/ Tamil	: Thanneer vittan kizhangu, ammaikodi.
Sanskrit	: Shatavari
Kannada	: Ashadhi
Marathi	: Shatavari mool
Telugu	: Pillipichara

Macroscopy

A loose network of hyphae accumulated at the root surface, and coils formed around root hairs and external to epidermal cells overlying short cells of the dimorphic, suberized exodermis.

Phytochemical constituent:

Asparagamine A, Polycyclic alkaloid, Two new steroidal saponins- shatavaroside A and B, Polysacchrides, Mucilage, Sterols and Isoflavone.

Therapeutic uses:

Treatment of gastric ulcer, dyspepsia, galactogogue, stomach spasms, nervous disorders, cancer, diarrhea, bronchitis, rheumatism, anti-inflammatory, diabetis, tuberculosis.

Dose: 16-32 mg/kg; Decoction- 50-100 ml.

5.3 Lippia nodiflora

Synonym : *Lippia nodiflora var*

Family : Verbenaceae

Part used : Leaves

Vernacular name

Sanskrit : Vasir vasuka

Hindi : Bhuiokra

Siddha/ Tamil : Podutalei

Marathi : Ratolia

Telugu : Bokkena

Kannada : Nelahippali

Macroscopy

Leaves arise in pairs at stem nodes and are rounded 10-20 mm long, 3-7 mm wide entire or bluntly toothed at the tip and narrow towards the petiole (2-5mm) at the leaf base, leaves having a grayish green appearance due to a covering of fine hairs on their surface.

Phytochemical constituent:

Tannins, Saponins, Flavonoids, Alkaloids, Phenolic Compound, Triterpenoid, Steroid, Nodifloretin, Beta Sitosterol Glycoside, Stigmasterol Glycoside, Nodifloridin A And B along with Maltose, Lactose, Fructose, Xylulose Etc.

Therapeutic uses:

Snake bite, Leaves are used in asthma, rheumatism and fever. Anti fungal, skin problem, diuretics, hepato-protective, anti oxidant, diabetic, hypolipidaemic.

Dose 30-150 mg

5.4 Oldenlandia heynei

Synonym : *Hedyotis herbacea L*
Family : Rubiaceae

Part used : Leaves

Vernacular name

Hindi : Paper-Bhed

Siddha/ Tamil : Nonganam Pillu

Sanskrit : Chhayaparpatika

Marathi : Papti

Telugu : Verri Nelavaeu, Thella Nela Vaemu

Kanna : Dakaag Purale

Macroscopy

Much branched, erect herbs, stem 4 angled, narrowly winged along angles.

Leaves sessile 1.5-3 × 0.1-0.3 cm, linear- lanceolate, acute at apex, glabrous, 3-4 setose on margins.

Phytochemical constituent:

Glycosides it contains Phenolic Glycoside, Flavanoidal Glycoside.

Therapeutic uses:

Bronchial asthma, rheumatism, varicose veins, syphills, gout, bone disorders, ulcer,fluid retention.

Dose: 100-200 mg.

5.5 Allium sativam L

Synonym : *Allium controversum*
Family : Liliaceae

Part used : Bulb

Vernacular name

Hindi : Lahsan

Siddha/ Tamil : Vellai poondu

Sanskrit : Arishtha

Marathi : Ghagra

Telugu : Korralu

Kannada : Belluli

Macroscopy

Rounded, composed of up to bout 15 smaller bulblets known as cloves. cloves and bulbs are covered by a whitish or pinkish tunic(papery coat)

Phytochemical constituent:

Allicin, Sulfur compound Allin, Peptides, Steroids, Terpenoids, Flavonoids, Phenols.

Therapeutic uses:

Anthelminic, increase appetite, Bronchitis, inflammation, piles, leucoderma, asthma, carminative, paralysis, pain in the body and joints, chronic fevers, High blood pressure treatment, warts treatment, Diabetes, upper respiratory tract infection, cholesterol treatment.etc.

Dose: 600- 1200 mg

5.6 Bacopa monnieri (L) Pennell

Synonym : *Lysimachia monnieri* (L)
Family : Scrophulariaceae

Part used : Leaves

Vernacular name

Hindi : Brahmi

Siddha/ Tamil : Nirbrahmi

Sanskrit : Jalasaya

Marathi : Brahmi

Telugu : Sambrani chettu

Kannada : Jalabrahmi

Macroscopy

A small, creeping herb, its stems are obtuse- angular, the leaves are short- petioled, cuneate to obovate, the capsules are ovoid. It can be easily grown in damp areas and can be propagated using seeds or vegetatively.

Phytochemical constituent:

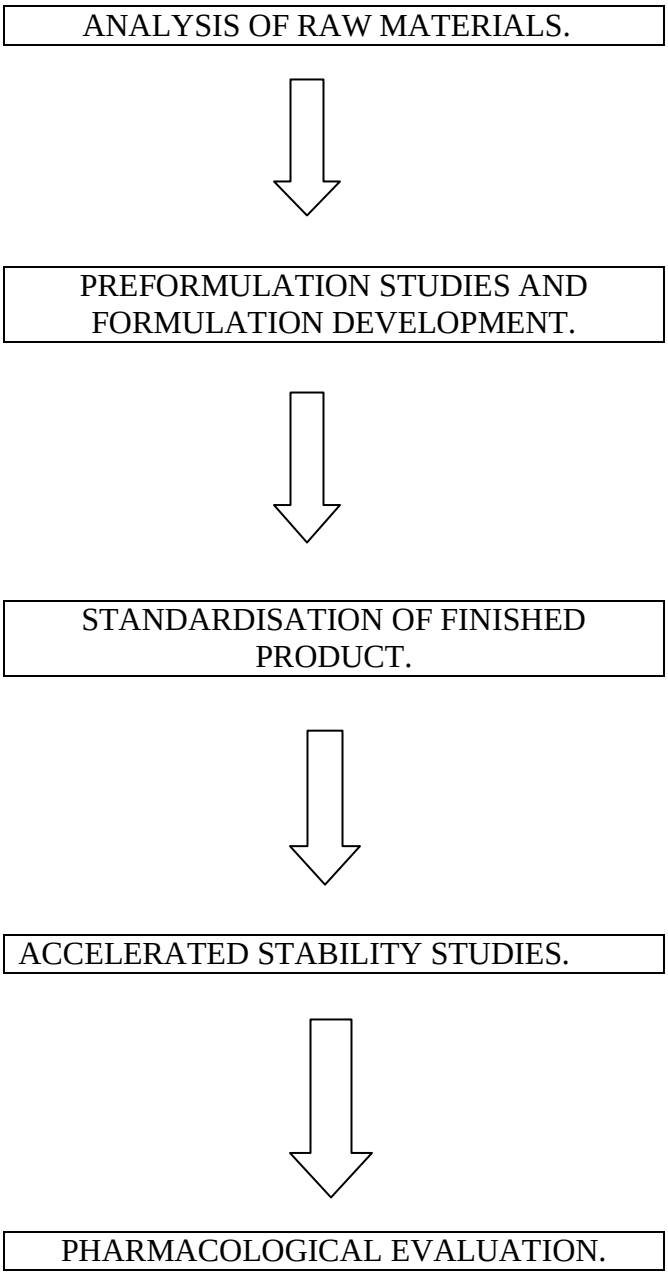
Alkaloid (Brahmine and Herpestine), Saponins, Flavonoids (Leuolin& Apigenin), Betulic acid, Stigmasterol, Beta sitosterol, Bacosaponins- Bacosaponin F&E, Bacopasiden,III,IV,V, Monnierin.

Therapeutic uses:

Epilepsy, asthma, ulcers, tumors, leprosy, anemia antioxidant, joint pain (rheumatism), Brain tonic, cardio tonic, diuretic.

Dose : 300 mg

6. PLAN OF WORK



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6.1 ANALYSIS OF RAW MATERIALS.

For the formulation of the polyherbal capsules, crude herbal drugs were used for powder in dry form.

- ***Asparagus racemosus***
- ***Allium sativum***
- ***Bacopa monnieri.***
- ***Lippia nodiflora***
- ***Oldenlandia heyneii***
- ***Smilax zylanica***

- **FOR CRUDE DRUG ANALYSIS**

- Foreign organic matter
- Loss on drying

Determination of Ash value

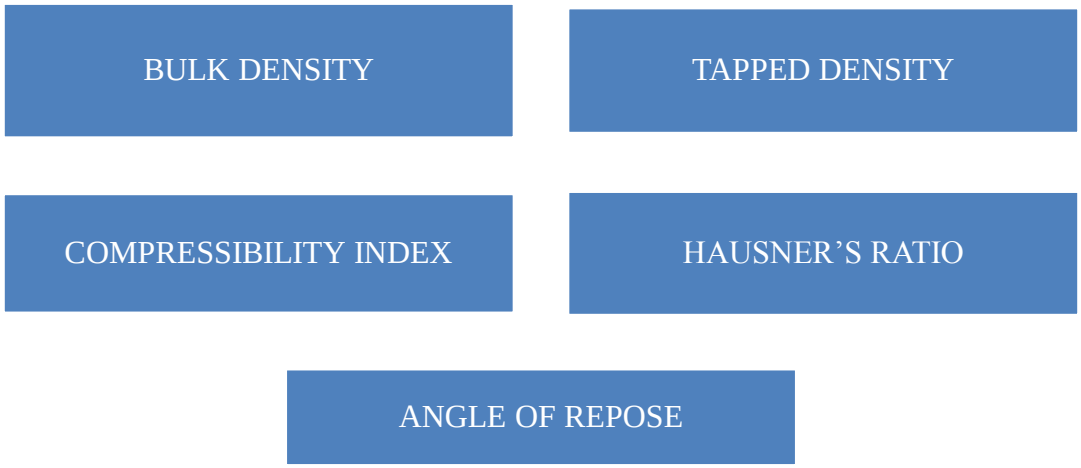
- Total ash
- Acid insoluble ash
- Water soluble ash
- Sulphated ash

Extractive value

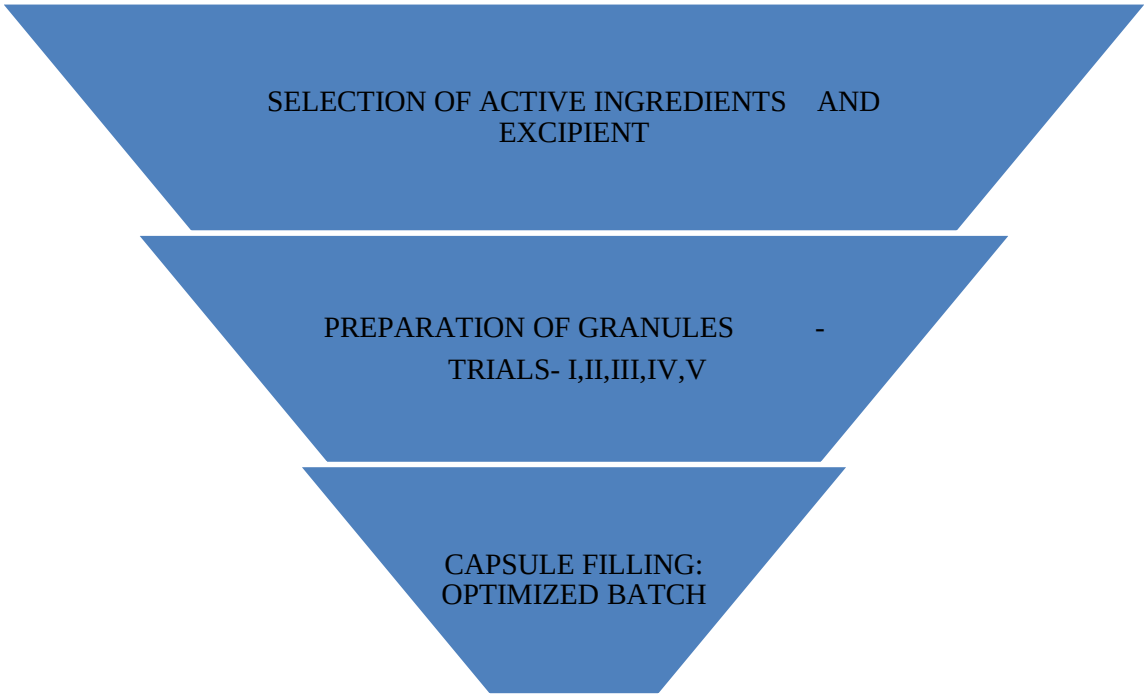
- Water soluble extractive
- Alcohol soluble extractive

Crude fiber content determination
Fluorescence analysis
Phytochemical studies
Heavy metal analysis
Microbial load analysis

PREFORMULATION STUDIES



FORMULATION DEVELOPMENT



6.2 STANDARDISATION OF FINISHED PRODUCT

- **Description**
- **Uniformity of weight**
- **Disintegration test**
- **Moisture content Determination**
- **pH**
- **Total ash**
- **Acid insoluble ash**
- **Water soluble ash**
- **Sulphated ash ash**
- **Water soluble extractive**
- **Alcohol soluble extractive**
- **Crude fiber content determination**
- **Preliminary phytochemical evaluation**
- **Quantitative estimation of phytoconstituent**
    - Total alkaloids content
    - Total saponins content
    - Total flavanoids content
    - Total tanins content
    - HPTLC Fingerprinting
    - Heavy metal analysis
    - Microbial load analysis
    - Determination of pesticide residue

6.3 ACCELERATED STABILITY STUDIES.

INITIAL STUDY	30 DAYS	60 DAYS	90 DAYS
Description Uniformity of weight Loss on drying Disintegration test PH Total ash Acid insoluble ash Water soluble ash Sulphated ash Water soluble extractive Alcohol soluble extractive Crude fiber content Total saponins content Total flavanoids content Total tanins content Total alkaloids content	Description Uniformity of weight Loss on drying Disintegration test PH Total ash Acid insoluble ash Water soluble ash Sulphated ash Water soluble extractive Alcohol soluble extractive Crude fiber content Total saponins content Total flavanoids content Total tanins content Total alkaloids content	Description Uniformity of weight Loss on drying Disintegration test PH Total ash Acid insoluble ash Water soluble ash Sulphated ash Water soluble extractive Alcohol soluble extractive Crude fiber content Total saponins content Total flavanoids content Total tanins content Total alkaloids content	Description Uniformity of weight Loss on drying Disintegration test PH Total ash Acid insoluble ash Water soluble ash Sulphated ash Water soluble extractive Alcohol soluble extractive Crude fiber content Total saponins content Total flavanoids content Total tanins content Total alkaloids content

6.4 PHARMACOLOGICAL EVALUATION.

IN VITRO ANTI ARTHRITIC EVALUATION:

- Inhibition of Protein Denaturation.
- Protenase inhibitory assay
- Membrane stabilization

INVITRO ANTIOXIDANT EVALUATION OF CAPSULE

- Nitric oxide scavenging assay
- Reducing power assay

IN VIVO ANTIARTHRITIC EVALUATION

- Acute Toxicity Study
- Evaluation of anti arthritic activity using COMPLETE FRAUND'S ADJUVANT induced arthritic rats

7. MATERIALS AND METHODS

LIST OF MATERIALS AND THEIR USES IN FORMULATION:

The materials used in the study were as follows:

Table 7.1 List of materials and their uses in the formulation

Name of the Materials	**Manufacturer/Supplier**	**Category**
Asparagus racemosus	Absa Herbals, Chennai	Active Ingredient
Bacopa monnieri	Absa Herbals, Chennai	Active Ingredient
Lippia nodiflora	Absa Herbals, Chennai	Active Ingredient
Oldenlandia heyneii	Absa Herbals, Chennai	Active Ingredient
Allium sativam	Absa Herbals, Chennai	Active Ingredient
Smilax zylanica,	Absa Herbals, Chennai	Active Ingredient
Talc	New drugs and chemicals, Bangalore	Excipient
Dicalcium phosphate	Global medicines ltd, Gujarat	Excipient
Aerosil	Global medicines ltd, Gujarat	Excipient
Magnesium stearate	Global medicines ltd, Gujarat	Excipient
Sodium methyl paraben	Global medicines ltd, Gujarat	Preservative
Sodium benzoate	Global medicines ltd, Gujarat	Preservative

EQUIPMENTS USED FOR THE STUDY

The equipments/instruments used in the study were as follows:

Table 7.2 Equipments used for the study

S.NO	**Name of the Equipments**	**Manufacturer/suppliers**
1	Weighing balance	Shimadzu, Japan
2	Hot air oven	Industrial Heaters, Chennai
3	Muffle Furnace	Industrial Heaters, Chennai
4	Disintegration Test apparatus	Veego, Mumbai
5	Blender	Cadmach, Mumbai
6	Capsule filling machine	Pam Pharma and Allied Machinery Co.Pvt.Ltd
7	Digital pH meter	Symchrony, India
8	Soxhlet apparatus	Symchrony, India
9	Stability chamber	Technico india, Mumbai
10	UV spectrophotometer	Shimadzu, Japan
11	Atomic Absorbance Spectrophotometer (6300)	Shimadzu, Japan

7.1.1 ANALYSIS OF RAW MATERIALS:

Sampling of the raw materials.[52]

From each container or package selected, three original samples were taken with care to avoid fragmentation. Samples were taken from the top, middle and bottom of the container. In

the case of sacks and packages, the three samples were taken by hand, the first from a depth of not less than 10 cm from the top and the second and third from the middle and bottom after cutting into the side of the package. The three original samples were then combined into a pooled sample which was mixed carefully. The average sample was obtained by quartering the pooled sample.

Authentication using reference samples

The original samples taken were authenticated by comparing with the authentic samples of the raw materials.

7.1.2 FOR CRUDE HERBAL DRUGS[53]

For crude herbal drugs, determination of foreign organic matter, loss on drying, total ash, acid insoluble ash, water soluble ash, sulphated ash, water soluble extractive, alcohol soluble extractive, limit tests for heavy metals, microbial load analysis were analysed according to Ayurvedic Pharmacopoeial procedure.

Determination of foreign matter

100g of the drug sample was weighed and then it was spread out in a thin layer. The foreign matter was detected by inspection with the use of a lens. Foreign matter found were separated and weighed and the percentage was calculated.

$$\text{Foreign organic matter} = \frac{\text{Weight of the sample after inspection}}{\text{Weight of the sample before inspection}}$$

Determination of moisture content

The loss on drying test is important when the herbal substance are known to be hygroscopic. An excess of water in medicinal plant materials will encourage microbial growth, the presence of fungi, insects and deterioration. In modern pharmaceutical technology, the water content provides information concerning the shelf life and quality of the drugs.

About 10 g of the drug (without preliminary drying) was placed after accurately weighed (within 0.01g) in a tarred evaporating dish and dried at 105^{o}c for 5 hours and weighed. The drying was continued and weighed, until the difference between two successive weighings corresponds to not more than 0.25 % at one hour interval. Constant weight is reached when two

consecutive weighings after drying for 30 minutes and cooling for 30 minutes in a desicator show not more than 0.01 g difference.

$$\text{Moisture content (\%)} = \frac{\text{Final weight of the sample}}{\text{Initial weight of the sample}} \times 100$$

DETERMINATION OF ASH VALUE

Ash values of a crude drug is defined as the inorganic residue remaining after incineration, which simply represent inorganic salts, naturally occurring in drug or adhering to it or deliberately added to it as a form of adulteration. Hence the ash values are helpful in determining the quality and purity of the crude drug in the powdered form.

Determination of Total ash value

About 2 grams of sample was weighed in a tared platinum or silica dish and was incinerated at a temperature not exceeding 450^{o}c until the sample free from carbon, cooled and weighed. The ash obtained was weighed. The percentage of the total ash was calculated.

$$\text{Total ash value} = \frac{\text{Weight of residue obtained}}{\text{Weight of the sample taken}} \times 100$$

Determination of acid insoluble ash value

The total ash was boiled with 25 ml of dilute hydrochloric acid for 5 minutes, insoluble matter was collected on an ashless filter paper, washed with hot water and ignited, cooled in desiccators and weighed. The percentage of acid insoluble ash was calculated.

$$\text{Acid insoluble ash} = \frac{\text{Weight of residue obtained}}{\text{Weight of the sample taken}} \times 100$$

Determination of water soluble ash value

The water soluble ash obtained from the total ash was boiled for 5 minutes with 25 ml of distilled water insoluble matter was collected in an ashless filter paper, washed with hot water, and ignited for 15 minutes at a temperature not exceeding 450 ^{o}c. The percentage of water soluble ash was calculated.

$$\text{Water soluble ash} = \frac{\text{Weight of residue obtained}}{\text{Weight of the sample taken}} \times 100$$

Determination of sulphated ash value

Silica crucible was heated to redness for 10 minutes, allowed to cool in a desiccator and weighed. 2g of the substance, accurately weighed, in to the crucible, ignited gently at first, until the substance is thoroughly charred. Cool, moisten the residue with 1 ml of sulphuric acid, heated gently until white fumes are no longer evolved and ignite at 800 ^{o}c± 25 ^{o}c until all black particles have disappeared. The ignition was conducted in a place protected from air currents. The crucible was allowed to cool, add a few drops of sulphuric acid heat. Ignite as before, cool and weighed .

$$\text{Sulphated ash} = \frac{\text{Weight of residue obtained}}{\text{Weight of the sample taken}} \times 100$$

EXTRACTIVE VALUES

The extractive values obtained by exhausting the crude drugs with solvent are indicative of the approximate measure of their chemical constituents. Taking into consideration the diversity in chemical nature and properties of contents of drugs, various solvents used for determination of extractive values.

Determination of water soluble extractive value

5 g of the air dried coarsely powdered drug was macerated with 100 ml of ethanol (95%) in a closed flask for 24 hours, shaking frequently (or in a mechanical shaker) during 6 hours and allowed to stand for 18 hours. Rapidly filtered, taking precautions against loss of solvent,

evaporated 25 ml of the filtrate to dryness in a tarred flat bottomed shallow dish and dried at 105^{o}c to constant weight and weighed. The percentage of water soluble extractive was calculated.

$$\text{Water soluble extractive value} = \frac{\text{Weight of the dried extract}}{\text{Weight of the sample taken}} \times 100$$

Determination of alcohol soluble extractive value

Procedure for water soluble extractive was followed for the determination of alcohol soluble extractive but the solvent used is 90% ethanol instead of alcohol.

$$\text{Water soluble extractive value} = \frac{\text{Weight of the dried extract}}{\text{Weight of the sample taken}} \times 100$$

Determination of Crude fibre content

The crude raw material should be powdered and mixed thoroughly. About 2 gm (it should be in between 2 to 2.1 gm) of the powdered sample must be weighed accurately and taken in 250 ml of dil. Sulphuric acid solution in a long necked 500 ml beaker. Then the beaker is placed in a hot plate and a flat bottomed round flask with long neck containing distilled water is placed above the beaker in order to condense the vapours (Note: Should be take care that the 1st appearance of the first bubble, after that boil for half an hour).Then filter by using a clean cloth and wash the precipitate with hot water to remove the acid, then collect the precipitate by washing with the dilute sodium hydroxide solution in a long neck beaker. Then collect the precipitate by filtering in a clean cloth .Then take carefully all the precipitate and transfer it into a silica crucible. Dry it in hot air oven for 6-8 hours and then cool. Weighed and kept in muffle furnace for half an hour, cool and then weighed.

PRELIMINARY PHYTOCHEMICAL SCREENING [54]

The crude raw material was subjected to preliminary phytochemical tests as per Phytochemical Methods.

Alkaloids:

To the extract, added few drops of bismuth iodide solution (dragendorff's reagent), reddish brown color was observed, which indicates the presence of alkaloids.

Carbohydrates:

In a test tube containing ethanolic extract of powdered drug, added 2 ml of distilled water and 2 drops of freshly prepared 20% alcoholic solution of alpha napthol. Mixed well and added 2 ml of concentrated sulphuric acid along the side of the test tube. Formation of red violet ring was observed at the junction of two layers, which disappears on addition of excess alkali solution, which confirms the presence of carbohydrates.

Glycosides:

Extracted 200 mg of drug with 5 ml dil sulphuric acid by warming on a water bath, filtered it and neutralized the acid extract with 5% solution of sodium hydroxide. Added 1 ml of fehlings's solution A and B until it became alkaline and heated on a water bath for 2 mins. Formation of red precipitate was observed, which indicates the presence of glycosides.

Phenols:

Dissolved a small quantity of ethanolic extract of the drug with 2 ml of distilled water, added a few drops 10% aqueous ferric chloride solution. A blue or green color was produced, which indicates presence of phenols.

Proteins (Biuret's test)

To 1 ml of ethanolic extract of the drug, 5 to 8 drops of copper sulphate solution (10%) was added. Formation of violet color was observed, which indicates the presence of proteins.

Saponins

To 5 ml of ethanolic extract of the drug, added a few drops of sodium bicarbonates solution. Shake the mixture vigorously and left for 3 minutes. Honey comb like froth developed, which indicates the presence of saponins.

Tannins

The substance was mixed with basic lead acetate solution formation of white precipitate was observed, which indicate presence of tannins.

Steroids

Treated the extract with few drops of acetic anhydride, boiled and cooled, and added concentrated sulphuric acid from the side of the test tube. A brown ring was formed at the junction two layers and upper layer turns green, which shows presence of steroids.

Flavones (shinoda test)

To the extract in alcohol few magnesium turnings and few drops of concentrated hydrochloric acid were added and boiled for 5 minutes and red coloration was observed, which shows for the presence of flavones.

Triterpenoids

Treated the extract with few drops of con.sulphuric acid , formation of yellow color was observed, which shows the presence of triterpenoids.

FLUORESCENCE ANALYSIS [55]

Samples were studied for any color changes with different chemicals and solvents. The samples were observed under UV chamber in different wavelengths viz., 254 nm (short wavelength) , ordinary light and 366 nm.(long wavelength).

HEAVY METALS ANALYSIS [56]

The formulation was analyzed for its heavy metals limits.

Preparation of samples and standards by acid digestion method

Accurately weighed 2 g of sample was taken in Kjeldahl flask. Acid mixture of HNO_3:$HClO_4$ (4:1) was added in the flask and heated continuously till the solution was colourless. The sample was then transferred in a 25 ml volumetric flask and the volume was made-up with distilled water. Reagent blank was synchronously prepared according to the above procedure. The standards of Lead (Pb), Arsenic (As) and mercury (Hg) were prepared as per the protocol and the calibration curve was developed for each of them. Then samples were analyzed for the presence of Lead, Arsenic and mercury using Atomic Absorbance Spectrophotometer (AAS) (6300 SHIMADZU).

MICROBIAL LOAD ANALYSIS:

The following test are carried out for the estimation of number of viable aerobic microorganisms present and for detecting the presence of designated microbial species in the herbal medicines.

1. *Total aerobic viable count*
2. *Yeast and moulds*
3. *Escherichia coli*
4. *Salmonellae*
5. *Shigella*
6. *Streptococcus*

7.2 PREFORMULATION STUDIES[57]

Prior to the development of the major dosage forms, it is essential that fundamental Physical and chemical properties of the drug molecule and other derived properties of the drug Powders are determined. This information decides many of the subsequent events and approaches in formulation development. This first learning phase is known as Preformulation.

Definition:

Preformulation involves the application of biopharmaceutical principles to the Physicochemical parameters of drug substance are characterized with the goal of designing Optimum drug delivery system. Before beginning the Preformulation programs the Preformulation scientist must consider the following factors:

- The amount of drug available.
- The physicochemical properties of the drug already known.
- Therapeutic category and anticipated dose of compound.
- The nature of information, a formulation should have or would like to have.

7.2.1 DETERMINATION OF THE GRANULES PARAMETERS[58]

- Bulk density
- Tapped density
- Compressibility index
- Hausner's ratio
- Angle of repose

Bulk density (ρb)

It is determined by measuring the volume of a known mass of powder sample that has been passed through a screen into a graduated cylinder or through a volume measuring apparatus into a cup. It is expressed in g/ml and is given by,

$$\rho b = M/Vo$$

Where, M - is the mass of powder

V_o- is the bulk volume of the powder.

The inter particle interactions that influence the bulking properties of a powder are also the interactions that interfere with powder flow, a comparison of the bulk and tapped densities can give a measure of the relative importance of these interactions in a given powder. Such a comparison is often used as an index of the ability of the powder to flow.

Tapped density (ρt)

It is achieved by mechanically tapping a measuring cylinder containing a powder sample. After observing the initial volume, the cylinder is mechanically tapped and volume readings are taken until little further volume change is observed.

The mechanical tapping is achieved by raising the cylinder and allowing it to drop under its own weight at a specific distance.

The tapped volume was measured by tapping the powder to constant volume. It is expressed in g/ml and is given

$$\rho t = M/Vt$$

Where, M - Mass of powder and V_t- Tapped volume of the powder.

Compressibility index: (CI)

Compressibility is the ability of powder to decrease in volume under pressure. Compressibility is a measure that obtained from density determination. Weighed quantity of granules was transferred to 50 ml graduated cylinder, volume occupied by granules was noted down. Then cylinder was subjected to 500/ 750 and 1250 taps. The difference between two tabs should be less than 2%. The percentage Compressibility Index is calculated by using formula.

$$CI = \frac{Vo-Vi}{Vo} \times 100$$

Where, V_o- Untapped density; V_i- Tapped density

Hausner's Ratio

It is measurement of frictional resistance of the granular material. The Ideal range should be 1.2 -1.5, it was determined by the ratio of tapped density and bulk density.

$$\text{Hausner's Ratio} = V_i / V_o$$

Where, V_o -Untapped density, V_i -Tapped density

Angle of repose

The tangent of angle of repose is equal to the coefficient of friction between the particles. Hence the rougher and more irregular the surface of particles, the greater will be angle of repose. For determination of angle of repose (Θ), the blends were poured through the walls of a funnel which was fixed at a position such that its lower tip was at a height of exactly 2.0 cm above a hard surface. The drug or the blends were poured till the time when upper tip of the pile surface touched the lower tip of the funnel. Angle of repose was calculated using following equation.

The angle of repose Θ was calculated by the formula,

$$\text{Tan } \Theta = h/r,$$

$$\Theta = \tan^{-1} (h/r)$$

Where, Θ - angle of repose, h- height in cm and r- radius in cm.

Based on the Angle of repose, Compressibility index and Hausner's ratio, the flow property of the granules can be characterized.

Table 7.3 Angle of Repose, Compressibility Index and Hausner's Ratio[59]

Flow property	Angle of repose	Compressibility index	Hausner's ratio
Exacellent	25-30	<10	1.00-1.11
Good	31-35	11-15	1.12-1.18
Fair	36-40	16-20	1.19-1.25
Passable	41-45	21-25	1.26-1.34
poor	46-55	26-31	1.35-1.45
Very poor	56-65	32-37	1.46-1.59
Very very poor	>66	>38	>1.60

FORMULATION DEVELOPMENT STUDIES

7.2.2 SELECTION OF EXCIPIENTS[60]

For the formulation of capsules in addition to the active ingredients, excipients like diluents (filler), binder, disintegrating agent, lubricant and preservatives are required. The choice of excipients was made keeping in mind the current Food and Drugs Administration (FDA) regulations.

Diluents: Diluents/Fillers are added where the quantity of active ingredient is less (or) difficult to filling. Common tablet/capsule filler include Lactose, Dicalcium phosphate, Microcrystalline cellulose, etc.

Lubricants: They reduce friction during the filling process. In addition, they aid in preventing adherence of capsule material. Magnesium Stearate, Stearic acid, Hydrogenised vegetable oils and talc are commonly used lubricants.

Glidant: It is used to improve flow of the powder materials by reducing the friction between the particles. The most effective glidants are the Colloidal silicon dioxide, Talc and Starch.

Preservatives: The preservatives are added to herbal formulation to prevent contamination, deterioration and spoilage by bacteria, fungal and other microorganisms. The most effective preservatives are the sodium methyl paraben, sodium propyl paraben, sodium benzoate and bronopol.

EXCIPIENTS USED:

DILUENTS / FILLERS : Starch, Lactose, Magnesium stearate.
GLIDANTS : Talc, colloidal silicon dioxide, starch
PRESERVATIVES : Sodium methyl paraben, Sodium benzoate

GRANULATION [61]

Granulation is defined as a process of size enlargement, widely used in pharmaceutical, food, chemical, agriculture, animal feed and other industries in which powder particles are made to form larger, multi particle entities called granules. Pharmaceutical granules typically have a Size range between 0.2-0.4 mm, depending on their subsequent use. It is one of the most important unit operations for powder handling in pharmaceutical industry. The appearance, elegance and ease of filling of capsules are dependent on variety of variables like materials used, processing techniques and equipment used for ultimate quality of granules produced.

Table 7.4 Formula for granule preparation

ACTIVE INGREDIENTS	**QUANTITY mg/ capsule**
Asparagus racemosus	20mg
Bacopa monnieri	1.5mg
Lippia nodiflora	110mg
Oldenlandia heyneii	100mg
Allium sativam	15mg
Smilax zeylanica	100mg

PREPARATION OF GRANULES:

Step 1: All the individual herb plant materials were cleaned and then washed with demineralized water for 3-4 times and dry with shade.

Step 2: Each dried plant material was pulverized and passed through sieve 30 slotted stainless steel mesh.

Step 3: Plant ingredients weighed individually and pulverized into a moderately fine powder in a stainless steel pulveriser then mixed along with the binder and other excipients. To get uniform mixing.

Step 4: After, QA approval the granules were filled in "0" size red capsule with average content weight at 520mg.

SCHEME: POLYHERBAL CAPSULE FORMULATION

Formulation Development of polyherbal Capsules:

Active materials + Inactive materials

↓

Sifting

Mixing

Blend (Evaluation of Bulk density, Tapped density, Angle of repose, Compressibility Index, Hausner's ratio)

Capsule filling (Uniformity of weight, DisintegrationTest)

7.3 Trial batches: (Size: 500 Capsules)

Various trial batches (size: 500 capsules) were formulated by varying the composition of the excipients proportions. Based on the various excipients proportions five trial batches given in Table 7.5

Table7.5 Trial batches (Size: 500 capsules)

Materials	**Trial- 1**	**Trial- 2**	**Trial- 3**	**Trial- 4**	**Trail- 5**
Asparagus racemosus	20 mg	20 mg	20 mg	20 mg	20 mg
Lippia nodiflora	110 mg	110 mg	110 mg	110 mg	110 mg
Olden landia heynaii	100 mg	100 mg	100mg	100 mg	100 mg
Smilax zylanica	100 mg	100 mg	100 mg	100 mg	100 mg
Allium sativam	15 mg	15 mg	15 mg	15 mg	15 mg
Bacpa monnieri	1.5 mg	1.5 mg	1.5 mg	1.5 mg	1.5 mg
Talc	100 mg	-	100 mg	100 mg	100 mg
Aerosil	1 mg	1mg	-	1 mg	1 mg
Dicalcium phasphate	-	100 mg	50 mg	50 mg	50 mg
Magnesium sterate	-	-	2 mg	-	1.25 mg
Sodium Methyl Paraben	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.5 mg
Sodium benzoate	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.5 mg

From the above trial batches, the trial batch 5^{th} trial was found to be the perfect batch and it was selected for the consideration of further large scale manufacturing.

FINAL TRIAL BATCH SIZE: 5000 CAPSULES:

Table7.6 Final trial batch (Size: 5000 Capsules)

S.NO	Ingredient	Quantity (mg/capsules)
1	*Asparagus racemosus*	20 mg
2	*Lippia nodiflora*	110 mg
3	*Olden landia heynaii*	100 mg
4	*Smilax zylanica*	100 mg
5	*Allium racemosus*	15 mg
6	Bacopa monnieri	1.5 mg
7	Talc	100 mg
8	Aerosil	1 mg
9	Dicalcium phasphate	50 mg
10	Magnesium sterate	1.25 mg
11	Sodium Methyl Paraben	0.5 mg
12	Sodium benzoate	0.5 mg

Fig.7.1 Formulated capsules

CAPSULE FILLING

- The formulated granules were filled in "0" size capsules to an average net content weight of 520 mg.
- The capsules were then dedusted, transferred into polybags, labelled and the samples were evaluated as per the testing requirements.
- After approval from QAD the capsules were packed as per the packing instructions.
- From the final trial, samples were taken for accelerated stability studies as per the Testing requirements.

7.4 STANDARDISATION OF FINISHED PRODUCT[62,63]

The developed capsules were subjected to following studies for their standardization:

- Evaluation of capsules

- Physicochemical parameters
- Phytochemical studies
- Heavy metal analysis
- Microbial load analysis

7.4.1 EVALUATION OF CAPSULES

- Description
- Uniformity of weight
- Disintegration test
- Moisture content

Description

The general appearance of a capsule, its visual identity and overall "elegance" is essential for consumer acceptance. The color, shape, odor and surface texture are all noted for the capsules prepared.

Uniformity of weight

20 individual units were selected at random and their content was weighed and their Average weight was calculated. Not more than two of the individual weights deviate from the average weight by more than the percentage shown in the table 7.7

Table: 7.7 Acceptance Criteria I.P Limit

Dosage form	**Average weight**	**% Deviation**
Capsules	<300 mg	10 %
	>300 mg	7.5 %

Disintegration test

This test was done to measure the time taken by the formulation to disintegrate in a liquid medium. This is done to determine whether the capsule disintegrates within the prescribed time when placed in a liquid medium under the prescribed experimental conditions. One capsule each was added to each of the six tubes of the basket and a disc was added to each of the tube. The tubes were dipped in 0.1N HCl solution maintained at 37°C. The time was noted.

Determination of moisture content

The method was carried out as detailed in the 7.1.2 in this chapter given earlier.

7.4.2 PHYSICOCHEMICAL PARAMETERS

P^H

1 g of capsule powder was taken and dissolved in 100 ml demineralized water. The pH value of the solution was determined by means of a digital P^H meter. The pH meter was calibrated using buffers of 4, 9 and 7 P^H. The electrodes were immersed in the test solution and P^H was measured.

FOR POLYHERBAL FORMULATION

Determination of Foreign organic matter, Loss on drying, Total ash, Acid insoluble ash, Water soluble ash, Sulphated ash, Water soluble extractive, Alcohol soluble extractive, Microbial load analysis were analysed according to Ayurvedic Pharmacopoeial procedure.

CRUDE FIBRE CONTENT

The determination of crude fibre content was carried out as detailed in the 7.1.2 in this chapter earlier.

7.4.3 PHYTOCHEMICAL STUDIES

Preliminary Phytochemical examination

The preliminary phytochemical examination was carried out as detailed in the 7.1.2 in this chapter given earlier.

7.4.4 QUANTITATIVE ESTIMATION OF PHYTOCHEMICALS

Quantitative Estimation of Total Alkaloids[64]

5 g of the granules were weighed into a 250 ml beaker and 200 ml of 10 % Acetic acid in ethanol was added, covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to 1/4th of the original volume. Concentrated NH_4OH was added drop wise to the extract until the precipitation was completed. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH_4OH and then filtered. The residue was dried and weighed.

Quantitative Estimation of Total Saponins

20 g of the granules were weighed and 100 ml of 20 % Ethanol was added. Then the sample was heated over a hot water bath for 4 hours with continuous stirring at about 55º C. The mixture was filtered and the residue re-extracted with another 200 ml 20 % ethanol. The combined extract was reduced to 40 ml over water bath at about 90º C. The concentrate was treated with 20 ml of diethyl ether and the aqueous layer was recovered while the ether layer was discarded. This process of purification was repeated three times and then 60 ml of n- Butanol was added and extracted. The n-Butanol extract obtained was then washed two times with 10 ml of 5% Aqueous sodium chloride. The remaining solution was heated in a water bath for evaporating the solvent. After evaporation the samples were dried in the oven to a constant weight and the Saponin content was calculated as percentage.

Quantitative estimation of Total Tannins[65]

Tannin content present in the formulation was estimated by Tocklai-Lowenthal method. This is basically an Oxidimetric titration of the infusion of the sample followed by a similar determination on another portion from which tannin has been quantitatively removed. The difference between the "Total" and the "Non-Tan" titres gives the tannin titre. The infusion of the sample of extract was prepared by boiling 5 g sample with 400 ml water for 1 hour, cooling and making up the volume of the extract to 500 ml. The separation into tannins and non-tans was effected by gelatin. The following reagents were used.

1. Gelatin solution:

25 g gelatin was soaked for 1 hour in Saturated Sodium chloride solution. The mixture was then warmed until the gelatin got dissolved and after cooling the solution was made up to 1litre with Saturated Sodium chloride.

2. Acid Sodium chloride:

25 ml of conc. Sulphuric acid was added to 975 ml Saturated Sodium chloride solution. 100 ml of the sample infusion was mixed with 50 ml of the gelatin solution, 100 ml of acid Sodium chloride and 20 g of powdered Kaolin. After shaking for several minutes and allowing settling, the mixture was filtered and an aliquot of the filtrate withdrawn for titration. 25 ml of the non-tan filtrate is equivalent to 10 ml of the original infusion.

The Tocklai - Lowenthal method:

The following reagents were used:

(1) N/25 Potassium permanganate;

(2) 1-5 g Indigo carmine dissolved in 1litre water containing 50 ml Sulfuric acid.

A 10 ml aliquot of the infusion is mixed with 25 ml of the Indigo-carmine solution and the mixture diluted to 750 ml Potassium permanganate was then run in from a burette 1 ml at a time with brisk shaking. As the titration proceeded, the blue of the Indigo-carmine passed through many shades to a final yellow with a faint pink tint at the rim. This was taken as the endpoint.

(Note: This needs considerable judgement on the part of the operator. The titre is affected to some extent by the amount of tannin in solution, the time taken in the titration and the vigor of shaking. Repeats often differ by 0-3 ml or more and the variation between different operators may amount to 1 ml.) The tannin titre was arrived at by subtracting the non-tan from the total titre. The equivalent weight factor is 0.0416.

Quantitative estimation of total flavonoids[66]

10g of the sample was extracted repeatedly with 100ml of 80% Aqueous Methanol at room temperature. The whole solution was filtered through Whatman filter paper No.42

(125mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight.

7.4.5 CHROMATOGRAPHIC FINGERPRINTING OF HERBAL PRODUCTS [67]

Chromatographic fingerprinting has been in use for a long time for single chemical entity drug substances. Recently it has become one of the most powerful tools for quality control of herbal medicines. The use of chromatographic fingerprinting for herbal drugs tends to focus on identification and assessment of the stability of the chemical constituents observed by various chromatography techniques such as HPLC, TLC, HPTLC, GC, capillary electrophoresis.

HPTLC Finger printing of polyherbal capsules:

Chromatographic conditions:

Instrument	: CAMAG HPTLC
HPTLC Applicator	: CAMAG LINOMAT IV
HPTLC Scanner	: CAMAG TLC SCANNER II
Method	: as per AHRF Method
Mobile phase	: Ethyl acetate: Hexane (6:4)
Stationary phase	: HPTLC Silica MERCK 60F 254
Sample dilution	: 10 mg of sample dissolved in 1 ml of ethyl acetate
Vol. Of sample loaded	: 20 µl
Lambda max	: 254 nm
Detection	: R_f value was calculated, peak area of each band was detected.

7.4.6 HEAVY METALS ANALYSIS[67]

The formulation was analyzed for its heavy metals limits. Discussed earlier (7.1.2)

7.4.7 DETERMINATION OF PESTICIDAL RESIDUE BY TLC[68]

Pesticide residues generally accumulate from agricultural practices of spraying, treating soils during cultivation and through the administration of fumigants during storage. Since many medicinal preparations of plant origin are taken over long periods of time, limits for pesticide residues should be established, following recommendations of the Food and Agriculture

Organization (FAO) of the United Nations and World Health Organization (WHO) which have already established the safety index of these residues in food and animal feed. These guidelines include the analytical methodology of Pesticidal residues. Out of various pesticides available, only chlorinated hydrocarbons and related pesticides like aldrin, BHC, Chlordane, Dieldrin, DDT and a few Organo-phosphorus pesticides like Carbophenothion retain a long residual action.

Extraction of common pesticide from material:

10 g of polyherbal capsule powder were taken in a round bottom flask and added Sodium sulfide with 100ml n-Hexane. It was refluxed for 1 hour and filtered. The filtrate extracted with 50ml and 25ml of Acetonitrile. The Acetonitrile layer was mixed with 500ml demineralized water with 2.5ml saturated sodium sulfide and then extracted with an n-Hexane layer and evaporated on a water bath. This residue was used for the analysis of Organochloro, Organophosphate and Carbamate pesticides by Thin layer chromatography using standard reference compounds (Accu standards, USA).

TLC details:

Sample solution	: Residue in Methanol
Development system	: Benzene : Methanol (60: 40)
Stationary Phase	: Silica gel 60 F254 TLC plate of 0.2mm thickness.
Detection	: By UV Absorption Range from 200 to 300nm.

The Extracts were spotted along with reference standards and chromatogram was developed and analyzed under UV from 200 to 300 nm.

7.5 ACCELERATED STABILITY STUDIES OF CAPSULES[69,70,71,72]

Definition

Stability is defined as the extent to which a product retains, within specified limits and throughout its period of storage and use (i.e., its shelf-life), the same properties and characteristics that it possessed at the time of its manufacture.

Essential conditions for stability studies:

The stability study shall be performed for the following conditions:

- Introduction of new products/new process.
- Change in the manufacturing process/critical process parameter.
- Change in vendor of an active raw material, Change in facility/location.
- Reprocessing for the first time.
- Change in the packaging mode/new packs.
- For regulatory purposes.
- Change in the storage condition.

Conditions of Stability studies

Accelerated condition of 40° C ± 2° C / 75% RH ± 5% RH

- Long term condition of 25° C ± 2° C / 60% RH ± 5 % RH
- Long term / intermediate condition of 30° C ± 2° C / 75 % RH ± 5% RH

The ICH Harmonized Tripartite Guideline provides a general indication on the requirements for stability testing of new drug substances and products. The main thrust of the stability guideline centers on criteria for setting up stability protocols.

Climatic zones: The four zones in the world that are distinguished by their characteristic prevalent annual climatic conditions.

Table: 7.8 Climatic zones and derived storage conditions (± 2°C / ± 5% RH)

Zone	Condition	Temperature	Humidity
Zone I	Temperate	21°C	45% RH
Zone II	Subtropical, with possible high humidity	25°C	60% RH
Zone III	Hot/dry	30°C	35% RH
Zone IV	Hot/humid	40°C	70%RH

As our country comes under zone IV, the following conditions were maintained throughout the study

Table:7.9 Accelerated stability condition

Study	Storage condition	Time period covered
Accelerated stability study	40°C ±2°c	3months

The stability study of capsules under accelerated conditions was done according to the ICH guidelines and the duration of study were 6 months. The conditions of temperature and relative humidity were maintained 40° C ± 2° C / 75% RH ± 5% RH respectively in the stability chamber.

THE PARAMETERS STUDIED WERE:

- Description
- Uniformity of weight
- Disintegration test
- Moisture content
- pH
- Total ash
- Acid insoluble ash
- Water soluble ash
- Sulphated ash
- Water soluble extractive
- Alcohol soluble extractive
- Crude fibre content
- Quantitative estimation of Total Alkaloids
- Quantitative estimation of Total Saponins
- Quantitative estimation of Total Flavanoids
- Quantitative estimation of Total Tannins
- Microbial load analysis

    Total aerobic bacteria

    Yeasts and Moulds

    Escherichia coli

    Salmonella

Shigella

Streptococcus

The samples were analyzed for all the parameters except microbial load analysis at the beginning of the study and at the end of 30 day, 60 days and 90 days.

PHARMACOLOGICAL EVALUATION

Preparation of extract:

Polyherbal capsule powders were successfully extracted with hydro alcoholic mixture (3:2) for 10hrs using soxhlet method. Then the hydro alcoholic extract was centrifuged twice at 1500rpm for 5 min. Then the extract was subjected to evaporation on water bath to dryness. The resulted dried extract was used for the *in vitro* antioxidant and antiarthritic activity.

7.6 IN *VITRO* ANTIARTHRITIC ACTIVITY[73,74,75,76]

7.6.1 INHIBITION OF PROTEINASE ENZYME ACTIVITY

Principle

Neutrophils are known to be a rich source of proteinase. They carry many neutral serine Proteinase in their lysosomal granules. It was previously reported that leucocyte proteinase play an important role in the development of tissue damage during inflammatory reactions. Trypsin is a serine protease found in the digestive system of many vertebrates, where it hydrolyses proteins. In this assay trypsin is used as protease enzyme and casein is used as substrate.

Requirements

- Trypsin
- 25mM Tris HCl buffer (PH 7.4)
- 0.8%w/v Casein
- 70% v/v Perchloric acid

Procedure

- The reaction mixture (2.0 ml) contains 0.06 mg trypsin, 1.0 ml of 25 mM Tris-HCl buffer (pH 7.4) and 1.0 ml Aqueous solution of test sample (100, 200, 400,800 mcg/ml) and were incubated at 37ºC for 5 minutes.
- Then 1.0 ml of 0.8% (w/v) Casein was added and incubated for 20 minutes.
- 2.0 ml of 70% (v/v) Perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged.
- Optical density of the supernatant was read at 280 nm against buffer as blank. The percentage of inhibition was calculated using the following formula.

$$\text{Percentage inhibition} = \frac{(\text{O.D. of control} - \text{O.D. of sample})}{\text{O.D. of control}} \times 100$$

7.6.2 PROTEIN DENATURATION ASSAY

Principle

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well-documented cause of inflammation in RA. In this assay the proteins denaturation was induced by heat.

Requirements

- 5% Aqueous solution of Egg albumin
- Phosphate buffer saline (P^H 6.3)
- 1N HCl

Procedure:

- The reaction mixture (5ml) consists of 0.05ml of test sample (100, 200,400,800mcg/ml) and 4.5ml egg albumin (5% aqueous solution), pH of the reaction mixture was adjusted at 6.3 using small amount of 1N HCl.
- Then incubated at 37 ºC for 20 min and then heated to 51º C for 20 min. After cooling the samples, the turbidity was measured at 660nm.

- The experiment was performed in triplicate. The percentage inhibition of protein denaturation was calculated as follows

$$\text{Percentage inhibition} = \frac{(\text{O.D. of Control} - \text{O.D. Sample})}{\text{O.D. of Control}} \times 100$$

Control represents 100% protein denaturation. The results were compared with standard (Diclofenac sodium 200 mcg/ml) sample.

7.6.3 Membrane stabilizing assay

Principle

The erythrocyte membrane resembles to lysosomal membrane and as such the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. Therefore, as membrane stabilizes that interfere in the release or action of mediators like histamine, serotonin, prostaglandins, leukotrienes etc. The principle involved in this assay is stabilization of rat red blood cell membrane against hypo tonicity induced membrane lysis.

Requirements

- Hypotonic saline (0.25% NaCl)
- Phosphate buffer (pH 7.4)
- 10% rat RBC in normal saline

Procedure

- The reaction mixture (4.5 ml) consisted of 2 ml hypotonic saline (0.25% NaCl),1 ml 0.15 M phosphate buffer (pH 7.4) and 1 ml test sample (100, 200, 400 and 800 mcg/ml of final volume) in normal saline. 0.5 ml of 10% rat RBC in normal saline was added.
- For control sample, 1 ml of isotonic saline was used instead of test solution and sample solution without RBC act as product control.
- The mixture was incubated at 56º C for 30 minutes. The tubes were cooled under running tap water for 20 minutes. The mixtures were centrifuged and the absorbance of the supernatant was read at 560 nm.

Percentage membrane stabilizing activity was calculated as follows:

$$\text{Percentage Stabilization} = \frac{100 - (\text{O.D. of test} - \text{O.D. of Product control})}{\text{O.D of Control}} \times 100$$

The result was compared with standard (diclofenac sodium 200 mcg/ml) sample.

7.7 IN VITRO ANTIOXIDANT ACTIVITY[77,78,79]

7.7.1 Reducing power assay

Principle

The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation.

Requirements:

- Potassium ferricyanide
- Phosphate buffer
- Trichloroacetic acid

Procedure

Assay of Reducing Power was carried out by potassium ferricyanide method. 2.5 ml of various concentrations of test sample were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [$K_3Fe(CN_6)$] (10g/L). The mixture was then incubated at 50°C for 20 minutes. To this mixture 2.5 ml of trichloroacetic acid (100g/L) was added, which was then centrifuged at 3000 rpm for 10 minutes. Finally 2.5 ml of the supernatant solution was collected and mixed with 2.5 ml of distilled water and 0.5 ml $Fecl_3$ (1g/L) and absorbance was measured at 700 nm. Ascorbic acid was used as standard and phosphate buffer as blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power.

$$\% \text{ Reductive ability} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

7.7.2 Nitric oxide radical scavenging assay

Principle

Nitric oxide was generated from sodium nitroprusside which at physiological pH liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions (NO_2^-) which diazotize with sulphanilic acid and couple with napthylethylenediamine (Griess reagent) producing pink colour which can be measured at 546 nm.

Requirements:

- Sodium nitroprusside
- Griess reagent

Procedure

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5 M) in phosphate–buffered saline (PH 7.4) was mixed with 3ml of various concentrations of polyherbal formulation and incubated at 25 ^{o}C for 150 min. The samples from the above were reacted with Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with Sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm. Ascorbic acid has taken as positive control.

$$\text{Nitric oxide Scavenging (\%)} = \frac{\text{(Absorbance of Control - Absorbance of Sample)}}{\text{(Absorbance of Control)}} \times 100$$

7.8 IN VIVO ANTIARTHRITIC EVALUATION:

ACUTE TOXICITY STUDY[80,81]

Acute toxicity study was designed as per the OECD guidelines 423. (Acute toxic class method).

Principles and purposes

Acute toxicity testing determines the toxicity of a chemical or drug substances after single administration.

The main purpose of acute toxicity study is to evaluate the degree of toxicity in a quantitative and qualitative manner with the purpose of comparing it with other drug substances (e.g. other drug candidates for the same indication). Further acute toxicity testing provides information about the acute toxicity testing effects of a chemical in a quantitative manner i.e. it generate information about mechanism of acute toxicity.

The method of determination has changed in the last three decade mainly for animal welfare reasons producing mortality in animals in order to determine LD 50 is no longer the main purpose of acute toxicity testing. The test is based on stepwise procedure with use of minimum number of animals per step. Sufficient information is obtained on the acute toxicity of the substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined dose. The substance is tested using a stepwise procedure, each step using three animals or a single sex (normally females). Absence or presence of compound related mortality of the animals dosed at one step will determine the next step i.e.

- No further testing is needed
- Dosing of three additional animals with the same dose
- Dosing of three additional animals with the next higher (or) the next lower dose levels

Experimental animals:

Healthy adult non pregnant female wistar albino rats weighing between 150-200g were selected for the study. For all the three animals water was provided ad libitum and food was withheld overnight prior to dosing.

Selection of dose levels and administration of doses:

Being a traditional herbal medicine, the mortality was unlikely at the highest starting dose level (2000mg/kg body weight). Hence a limit test at one dose level of 2000mg/kg body weight was conducted in all the three animals.

Observation:

The animals were observed individually after dosing once during the first 30 minutes, periodically for the first 24 hours, with special attention given during the first 4 hours, and daily thereafter for a total of 14 days. The following clinical observation were made and recorded.

- Toxic signs:

  All rats were observed for any toxic signs

- Body weight:

  Individual body weight was recorded for all the animals.

- Cage side observation:

  The faeces colour, faeces consistency, change in skin and fur, eyes, mucus membrane (nasal) of the animals were observed.

- Physical Examination:

  Physical observation included changes in respiratory system (rate), cardiovascular system (heart rate), autonomic nervous system (salivation, lacrimation, piloerection, urinary incontinence and defecation), central nervous system (drowsiness, convulsions, motor activity, writhing, motor in coordination, righting reflex, pinna reflex, corneal reflex, tremors were recorded.

7.9 IN VIVO EVALUATION OF ANTIARTHRITIC ACTIVITY [82,83,84]

The protocol for conducting the *in vivo* study in female adult albino wistar rats was approved by the Institutional Ethical Committee (IEC) of the Madras Medical College, Chennai-600003, India Approval no.vide 2/243/CPCSEA. Dated 22.11.13

Experimental design

Induction of Arthritis : ( )

Arthritis was induced by a single sub-planter injection of 0.1 ml of Complete Freund's adjuvant (Sigma Chemicals, USA) containing 1.0 mg dry heat-killed *Mycobacterium tuberculosis* per milliliter sterile paraffin oil into a foot pad of the left hind paw of male albino wistar rats except vehicle control . The swelling in hind paws were periodically examined in each paw from the ankle using vernier caliper. Diclofenac sodium and Polyherbal capsules were administered orally in the form of freshly prepared Solution in distilled water.

Fig7.2 After induction of CFA

Fig-7.3 Oral administration of test and standard drugs

The animals received the following treatments:

Group I: Distilled water, 1 ml/kg/day, orally for 14 days as Vehicle control.

Group II: Distilled water, 1ml/kg/day, orally for 14 days as Arthritic control

Group III: Diclofenac sodium,13.5mg/kg/day, orally for 14 days as Standard

Group IV: Polyherbal formulation 200 mg/kg/day, orally for 14days as Test drug I

Group V: Polyherbal formulation 400 mg/kg/day, orally for 14 days as Test drug II

Measurement of paw swelling:

On 0th day, the left hind paw swelling of al rats was measured using vernier caliper and on 1st day arthritis was induced in all rats using CFA. The aforementioned drug treatment was started on 1st day and continued for 14 days. The assessment of antiarthritic activity was carried out by measuring change in paw swelling and body weight on 4th, 8th, 14th and 21st day after induction. The percent inhibition of paw swelling as determined as:

$$\frac{(V_C - V_0) - (V_t - V_0)}{(V_C - V_0)} X100$$

Where V_C is the paw swelling after induction,

V_0 is the paw swelling before induction,

V_t is the paw swelling after treatment.

Estimation of Hematological parameters

On the 22nd day , blood was withdrawn through retro-orbital vein puncture of all groups and the biochemical parameters such as Hemoglobin content, Total WBC count, ESR, RBC, PCV,DC were analyzed.

Radiographic study

Computer radiograph of arthritic induced Hock joint (ankle joint) and Stifle joints (knee joint) of treated and arthritic control group on 21st day were studied using Agfa CR Siemen 500 MA X ray machine systems. The region between the distal femur and the proximal tibia (including patella) was radiographed through lateral views for study and interpretation.

Histopathological examination

The animals were sacrificed by cervical dislocation and the ankle joints of hind limbs were removed and placed in 10% buffered formalin. The fixed tissues were then decalcified and the sections were stained with Hematoxylin and Eosin. Slides were reviewed for the evaluation

of histopathological changes like soft tissue swelling, bone demineralization, pannus formation, cartilage erosion and joint space narrowing.

Statistical Analysis:

Results were expressed as Mean ± SEM. The data was analyzed using one way analysis of variance (ANOVA) followed by Dennett's test. P values <0.01 were considered as Significant.

8. RESULTS AND DISCUSSION

8.1 ANALYSIS OF RAW MATERIALS

Authentication:

The raw materials used in the formulation were individually procured from the Qualified vendors and they were authenticated by Dr. P.Jayaraman, Director, Plant Anatomy and Research Centre, Tambaram, Chennai.

Organoleptic characters

Organoleptic character for the raw materials were done with samples taken from the suppliers. The results obtained and the standard values are given table 8.1.1

Table 8.1.1 Organoleptic characters

Name of the plant	Nature	Colour	Odour	Taste
Asparagus racemosus	Coarse powder	Yellowish brown	Characteristic	Bitter
Allium sativum	Fine powder	Brown	Characteristic	Astringent
Lippia nodiflora	Coarse powder	Greenish yellow	Characteristic	Bitter
Bacopa monneri	Coarse powder	Light brown	Characteristic	Bitter
Oldenlandia heyneii	Coarse powder	Greenish yellow	Characteristic	Tasteless
Smilax zeylanica	Coarse powder	Brown	Characteristic	characteristic

Preliminary quality control of the raw materials

The raw materials were sampled, authenticated and studied for their compliance to Preliminary qualitative standards as established by Indian Pharmacopoeia, Ayurvedic Pharmacopoeia of India and other standard references. For those raw materials, for which no official standard were available, in house (IH) standards were created and results were compared with them. The raw materials were divided into two category viz., Crude herbal drugs and Extracts of the crude drugs. Then, they were analyzed for the qualitative and quantitative standards. The results were compared with the standard references and observed for its compliance. Those materials which met the standards were taken for the formulation and development. All the tests were carried out as per the test methods detailed in the materials and methods part previously.

Foreign organic matter

Table 8.1.2 Foreign organic matter

S.No	Ingredients	Foreign organic matter	Acceptable limit(%w/w)
1	*Asparagus racemosus*	0.10 ± 0.10	NMT 1%
2	*Allium sativum*	0.12 ± 0.11	NMT 2%
3	*Lippia nodiflora*	0.05 ± 0.03	NMT 2%
4	*Bacopa monneri*	0.50 ± 0.01	NMT 2 %
5	*Oldenlandia heyneii*	0.11 ± 0.13	NMT 1%
6	*Smilax zeylanica*	0.05 ± 0.04	NMT 1%

The value are expressed as mean ±SD, (N= 3); NMT- Not more than, NLT- Not les than

Loss on drying

Loss on drying for the raw materials were done. The results obtained and the standared values are given table in 8.1.3

Table8.1.3 **Loss on drying**

S.No	Ingredients	Loss on drying (%w/w)	Acceptable limit (%w/w)
1	*Asparagus racemosus*	3.51 ± 0.38	NMT 10%
2	*Allium sativum*	6.10± 0.12	NLT 6 %
3	*Lippia nodiflora*	3.13± 0.32	NMT 4%
4	*Bacopa monneri*	2.43 ± 0.01	NMT12%
5	*Oldenlandia heyneii*	1.56 ± 0.30	NMT 2 %
6	*Smilax zeylanica*	0.35 ± 0.47	NMT 1%

The value are expressed as mean±SD,(N=3) NMT- Not more than; NLT- Not les than

Total ash value

Total ash content of raw materials was determined, the values obtained and their acceptable limits defined are given in table 8.1.4

Table 8.1.4 Total ash value

S.No	Ingredients	Total ash value (%w/w)	Acceptable limit(%w/w)
1	*Asparagus racemosus*	4.4 ± 0.50	NMT 15%
2	*Allium sativum*	3.5 ± 0.07	NMT 4%
3	*Lippia nodiflora*	2.1 ± 0.10	NMT 10%
4	*Bacopa monneri*	2.56 ± 0.12	NMT18%
5	*Oldenlandia heyneii*	5.13 ± 0.15	NMT 4 %
6	*Smilax zeylanica*	1.12 ± 0.30	NMT 0.6 %

The value are expressed as mean ± SD, (N=3); NMT-Not more than

Acid insoluble ash

From the total ash content, the acid insoluble ash content of individual raw materials was determined and results are enumerated in table 8.1.5

Table8.1.5 Acid insoluble ash

S.No	Ingredients	Acid insoluble ash (%w/w)	Acceptable limit (%w/w)
1	*Asparagus racemosus*	0.86 ± 0.26	NMT 3%
2	*Allium sativum*	0.56 ± 0.13	NMT 1%
3	*Lippia nodiflora*	3.4± 0.26	NMT 5%
4	*Bacopa monneri*	2.45± 0.09	NMT6%
5	*Oldenlandia heyneii*	9.23± 0.15	NLT 5%
6	*Smilax zeylanica*	0.6 ± 0.26	NMT 0.06 %

The value are expressed as mean±SD,(N= 3) NMT- Not more than; NLT- Not less than.

Water soluble ash value

From the total ash content, the water soluble ash content of individual raw materials was determined and results are enumerated in table 8.1.6

Table 8.1.6 water soluble ash

S.No	Ingredients	Water soluble ash (%w/w)	Acceptable limit(%w/w)
1	*Asparagus racemosus*	2.66± 0.49	NMT 5%
2	*Allium sativum*	1.23 ± 0.09	NMT 3%
3	*Lippia nodiflora*	1.46±.025	NLT 4%
4	*Bacopa monneri*	4.31± 0.12	NLT 15%
5	*Oldenlandia heyneii*	8.23± 0.41	NMT 10 %
6	*Smilax zeylanica*	4.33±0.20	NMT 7%

The value are expressed as mean±SD,(N=3)NMT- Not more than; NLT- Not less than

Sulphated ash value

Sulphated ash for the raw materials were determined and results are enumerated in table 8.1.7

Table8.1.7 sulphated ash

S.No	Ingredients	Sulphated ash (%w/w)	Acceptable limit(%w/w)
1	*Asparagus racemosus*	5.16 ± 0.20	NMT 6%
2	*Allium sativum*	1.23 ± 0.78	NMT 2 %
3	*Lippia nodiflora*	1.4 ± 0.36	NMT 4 %
4	*Bacopa monneri*	1.7 ± 0.65	NLT 2 %
5	*Oldenlandia heyneii*	9.0 ± 0.10	NMT 10 %
6	*Smilax zeylanica*	4.33 ± 0.20	NMT 5 %

The value are expressed as mean±SD,(N=3) NMT- Not more than; NLT- Not less than

Water soluble Extractive value

Extractive values for the raw materials were determined in water and the results are tabulated in table 8.1.8

Table 8.1.8 Water soluble Extractive

S.No	Ingredients	Water soluble Extractive (%w/w)	Acceptable limit(%w/w)
1	*Asparagus racemosus*	6.5 ± 0.4	NMT 20 %
2	*Allium sativum*	5.12± 0.03	NMT 5%
3	*Lippia nodiflora*	4.33 ± 0.45	NLT 4 %
4	*Bacopa monneri*	3.15 ±0.012	NLT 15%
5	*Oldenlandia heyneii*	3.53 ± 0.37	NMT 5%
6	*Smilax zeylanica*	1.7 ± 0.21	NMT 2%

The value are expressed as mean ± SD, (N=3); NLT- Not less than; NMT- Not more than

Alcohol soluble extractive value

Extractive values for the raw materials were determined in alcohol (90% ethanol) and the results are enumerated. Table 8.1.9

Table 8.1.9 Alcohol soluble extractive

S.No	Ingredients	Water soluble Extractive (%w/w)	Acceptable limit(%w/w)
1	*Asparagus racemosus*	5.36 ± 0.53	NMT15%
2	*Allium sativum*	1.01± 0.01	NLT 2.5%
3	*Lippia nodiflora*	11.23 ± 0.25	NLT 4 %
4	*Bacopa monneri*	8.12±0.12	NLT 6%
5	*Oldenlandia heyneii*	4.5 ± 0.4	NMT 5 %
6	*Smilax zeylanica*	2.0 ± 0.05	NMT 3%

The value are expressed as mean±SD,(N= 3) NMT- Not more than; NLT- Not less than

Crude fibre content

The crude fibre content for the raw material were determined(duch method) and the results are tabulated.Table 8.1.10

Table8.1.10 Crude fibre content

S.No	Ingredients	Crude fibre content (%w/w)
1	*Asparagus racemosus*	0.42 ±0.23
2	*Allium sativum*	1.03 ±0.08
3	*Lippia nodiflora*	0.49 ± 0.05
4	*Bacopa monneri*	0.91±0.09
5	*Oldenlandia heyneii*	0.58 ± 0.28
6	*Smilax zeylanica*	0.89 ± 0.11

The value are expressed as mean±SD,(N= 3)

PHYTOCHEMICAL ANALYSIS

The chemical tests for various Phyto constituents in the raw materials were carried out and the results were recorded and detailed in Table 8.1.11

Table 8.1.11Phytochemical analysis

S.No	Chemical constituent	*Asparagus racemosus*	*Bacopa monneri*	*Allium sativum*	*Olden landia heyneii*	*Lippia nidiflora*	*Smilax zeylanica*
1	Carbohydrates	–	–	–	–	+	–
2	Alkaloids	+	+	–	+	+	+
3	Steroids	+	+	+	–	+	+
4	Glycosides	–	–	–	+	+	–
5	Flavanoids	+	+	+	+	+	+
6	Tannins	–	–	–	–	+	+
7	Phenolic compounds	–	–	+	–	+	+
8	Proteins	–	–	–	–	–	+
9	Terpenoids	–	+	+	+	+	+
10	Fats and oils	–	–	–	+	–	–
11	Gums and Mucilage	+	–	–	–	–	–

\+ present

\- absent

FLUORESCENCE ANALYSIS

Table 8.1.12 Fluorescence characteristic of powdered samples of *Asparagus racemosus*

S.No	Treatment	Short UV	Ordinary light	Long UV
1	Powder	Pale yellow	Light brown	Light brown
2	Powder + water	colorless	Light brown	Light brown
3	Powder +Iodine	Dark green	Dark green	Dark green
4	Powder +Picric acid	Yellow	Dark yellow	Green
5	Powder + NaOH	Greenish yellow	Pale white	Colorless
6	Powder +Ethanol	Colorless	colorless	Colorless
7	Powder +KOH	Light brown	brown	Pale yellow
8	Powder +$FeCl_3$	Greenish yellow	brown	Black
9	Powder +Acetic acid	Colorless	Pale green	Colorless
10	Powder +Acetone	colorless	light brown	Light brown
11	Powder +Ammonia	Pale yellow	Light yellow	Colorless
12	Powder +HCl	colorless	brown	Colorless
13	Powder +H_2SO_4	Light yellow	Reddish brown	Colorless
14	Powder +HNO_3	Light brown	Light brown	Light brown

Table 8.1.13 Fluorescence characteristic of powdered samples of *Allium sativum*

S.No	Treatment	Short UV	Ordinary light	Long UV
1	Powder	Light brown	Light brown	Light brown
2	Powder + water	Brown	Light brown	Brown
3	Powder +Iodine	Black	Black	Black
4	Powder +Picric acid	Reddish brown	Brown	Reddish brown
5	Powder + NaOH	Light brown	Dark brown	Light brown
6	Powder +Ethanol	Colorless	Light brown	Colorless
7	Powder +KOH	Brown	Reddish brown	Brown
8	Powder +$FeCl_3$	Dark brown	Dark black	Dark brown
9	Powder +Acetic acid	Light brown	Black	Light brown
10	Powder +Acetone	Colorless	Light brown	Colorless
11	Powder +Ammonia	Light brown	Colorless	Dark brown
12	Powder +HCl	Colorless	Light brown	Light black
13	Powder +H_2SO_4	Reddish brown	Brown	brown
14	Powder +HNO_3	Brown	Colorless	Light brown

Table8.1.14 Fluorescence characteristic of powdered samples of *Lippia nodiflora*

S.No	Treatment	Short UV	Ordinary light	Long UV
1	Powder	Pale green	Green	Pale green
2	Powder + water	Pale green	Brown	Green
3	Powder +Iodine	Black	Black	Black
4	Powder +Picric acid	Pale green	Pale green	Pale green
5	Powder + NaOH	Dark green	Dark brown	Black
6	Powder +Ethanol	Pale green	Pale brown	Pale yellow
7	Powder +KOH	Yellowish green	Pale brown	Black
8	Powder +$FeCl_3$	Light green	Pale brown	Black
9	Powder +Acetic acid	Green	Dark green	Dark green
10	Powder +Acetone	Pale green	Dark green	Dark green
11	Powder +Ammonia	Pale green	Dark green	Dark green
12	Powder +HCl	Green	Light green	Yellowish green
13	Powder +H_2SO_4	Green	Light green	Yellowish green
14	Powder +HNO_3	Green	Light green	Yellowish green

Table 8.1.15 Fluorescence characteristic of powdered samples of *Bacopa monnieri*

S.No	Treatment	Short UV	Ordinary light	Long UV
1	Powder	Green	Green	Light green
2	Powder + water	Light green	Light green	Green
3	Powder +Iodine	Black	Black	Black
4	Powder +Picric acid	Light green	Yellowish green	Dark green
5	Powder + NaOH	Light green	Dark green	Light yellow
6	Powder +Ethanol	Dark green	Dark green	Black
7	Powder +KOH	Yellowish green	Black	Black
8	Powder +$FeCl_3$	Light green	Light green	Black
9	Powder +Acetic acid	Green	Yellowish green	Dark green
10	Powder +Acetone	Yellowish green	Brown	Dark green
11	Powder +Ammonia	Light green	Light green	Yellowish green
12	Powder +HCl	Green	Dark green	Yellowish green
13	Powder +H_2SO_4	Dark green	Dark green	Green
14	Powder +HNO_3	Dark green	Light green	Light yellow

Table8.1.16 Fluorescence characteristic of powdered samples of *Oldenlandia Heynei*

S.No	Treatment	Short UV	Ordinary light	Long UV
1	Powder	Pale green	Brown	Green
2	Powder + water	Green	Black	Black
3	Powder +Iodine	Black	Light brown	Dark green
4	Powder +Picric acid	Pale green	Light brown	Dark green
5	Powder + NaOH	Dark green	Dark brown	Black
6	Powder +Ethanol	Dark green	Dark brown	Black
7	Powder +KOH	Yellowish green	Light brown	Black
8	Powder +$FeCl_3$	Light green	Light brown	Dark green
9	Powder +Acetic acid	Light green	Light green	Black
10	Powder +Acetone	Yellowish green	Dark green	Dark green
11	Powder +Ammonia	Green	Black	Yellowish green
12	Powder +HCl	Green	Light green	Green
13	Powder +H_2SO_4	Light green	Light green	Light green
14	Powder +HNO_3	Light green	Light green	Light green

Table8.1.17 Fluorescence characteristic of powdered samples of *Smilax zeylanica*

S.No	Treatment	Short UV	Ordinary light	Long UV
1	Powder	Light brown	Brown	Brown
2	Powder + water	Brown	Dark brown	Dark brown
3	Powder +Iodine	Dark brown	Yellowish brown	Light brown
4	Powder +Picric acid	yellowish green	Light brown	Brown
5	Powder + NaOH	Green	Dark brown	Colorless
6	Powder +Ethanol	Green	Brown	Colorless
7	Powder +KOH	Colorless	Dark brown	Dark brown
8	Powder +$FeCl_3$	Light brown	Golden yellow	Dark brown
9	Powder +Acetic acid	Light brown	Brown	Brown
10	Powder +Acetone	Brown	Light brown	Colorless
11	Powder +Ammonia	Light green	Colorless	Black
12	Powder +HCl	Colorless	Colorless	Dark black
13	Powder +H_2SO_4	Colorless	Brown	Colorless
14	Powder +HNO_3	Light brown	Light brown	Light brown

LIMIT TEST FOR TOTAL HEAVY METALS

The limit test for heavy metals in the raw materials were carried out and the results were recorded and detailed in Table 8.1.18

Table 8.1.18 Limit test for heavy metals

S.No	Ingredients	Results	Standard
1	*Asparagus racemosus*	Complies	20 ppm Lead
2	*Allium sativum*	Complies	
3	*Lippia nodiflora*	Complies	
4	*Bacopa monneri*	Complies	
5	*Oldenlandia heyneii*	Complies	
6	*Smilax zeylanica*	Complies	

8.5 MICROBIAL LOAD ANALYSIS:

Microbial screening is done for the raw materials were carried out and the results obtained were detailed in Table 8.1.19

Table 8.1.19 Microbial load analysis

Ingredients	parameters	Results	Limits as per WHO
Asparagus racemosus *Allium sativum* *Lippie nodiflora* *Bacopa monneri* *Oldenlandia heynei* *Smilax zeylanica*	Total Microbial count	100cfu/g	NMT 1000cfu/g
	Yeast and mould	Nil	NMT 100 cfu/g
	Presence of *E.Coli*	Absent	Should be absent
	Presence of *Salmonella*	Absent	Should be absent
	Presence of *Shigella*	Absent	Should be absent
	Presence of *Streptococcus*	Absent	Should be absent

8.2 PREFORMULATION AND FORMULTION DEVELOPMENT STUDIES

Totally five trials of formulation were carried out using different choices of excipients Considering different facets of manufacturing problems as well as quality defects in mind. All the resultant formulations were evaluated for their flow property, uniformity of filling, uniformity of weight, moisture content and disintegration time.

Table8.2.1 Evaluation of trial batches

Parameters	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**
Bulk density(g/cm^2)	0.34±0.026	0.40±0.01	0.41±0.015	0.33±0.04	0.35±0.04
Tapped density(g/cm^2)	0.57±0.025	0.54±0.03	0.61±0.02	0.53±0.03	0.57±0.02
Compressibility index (%w/w/)	40.36±1.12	26.6±2.08	32±2.64	34.6±2.08	33±1.5
Housner's ratio	1.4±0.36	1.41±0.01	1.43±0.02	1.53±0.04	1.45±0.03
Angle of repose(degrees)	34.9±3.35	35.3±3.78	42±1.0	35±1.0	27.6±0.57

Mean ± Standard Deviation (n=3)

The trial 5 flow properties were within in the range of Excellent and they were in the Specified limits. So, fifth trial was chosen for further studies.

Table 8.2.2 Angle of Repose, Compressibility Index and Hausner's Ratio

Flow property	Angle of repose in degrees	Compressibility index (CI in %)	Housner's ratio
Excellent	25-30	<10	1.00-1.11
Good	31-35	11-15	1.12-1.18
Fair	36-40	16-20	1.19-1.25
passable	41-45	21-25	1.26-1.34
poor	46-55	26-31	1.35-1.45
Very poor	56-65	32-37	1.46-1.59
Very very poor	>65	>38	>1.60

From the data, it was observed that the final trial batch (Trial V) was the ideal one. This batch was considered for the optimization and pilot scale up study of that formula was done by exercising a large batch size viz., 5000 capsules instead of 500 capsules trials.

8.3 STANDARDISATION OF THE FINISHED PRODUCT

The final formulation was analyzed for its quality control parameters in three trials. The mean value was obtained and Standard deviation was calculated. Wherever there were no official standard, limits for each parameter was established based on trial and error analysis of Trial V capsules.

EVALUATION OF CAPSULES

1. Description

"light brown " coloured granules packed in "0" size Red capsules. The polyherbal capsules were evaluated for organoleptic characters which include colour, odour, taste and nature.

Table 8.3.1 **Organoleptic characters of capsules**

S.No	Parameters	Observations
1	Nature	Powder
2	color	Light brown
3	Odour	Slight aromatic
4	Taste	charecteristic

Uniformity weight of the capsule

Table 8.3.2Uniformity weight of the capsule

S.NO	Average weight/capsule(mg)	IP specification(mg)
1	515	**±7.5%**
2	510	
3	506	
Mean ± S.D	510.3±4.5	

Mean ± standard Deviation(n=20)

Disintegration Time

Table 8.3.3 Disintegration time

S.NO	Disintegration time(min)	IP specification(min)
1	10.21	NMT 30 Minutes
2	10	
3	11	
Mean ± S.D	10’9±0.5	

Mean ± Standard Deviation (n=3)

Capsules passed the disintegration test and the results complied as per the I.P.2010 specification.

Determination of Moisture Content

Table 8.3.4 Loss on drying

S.NO	LOD %w/w	IP specification
1	2.1	NMT 5%w/w
2	1.0	
3	2.2	
Mean±S.D	2.1±0.1	

Mean ± Standard Deviation (n=3)

PHYSICOCHEMICAL PARAMETERS

P^H of the 1%w/v aqueous solution of the formulation

Table 8.3.5 P^H

S.NO	P^H	IP Specification
1	5.61	5-6
2	5.82	
3	5.15	
Mean±S.D	5.52±0.68	

Mean ± Standard Deviation (n=3)

Determination of Total Ash

Table8.3.6 Total Ash

S.NO	Value(%w/w)
1	3.4
2	4.06
3	4.01
Mean ±S.D	3.82±0.36

Mean ± Standard Deviation (n=3)

Acid insoluble ash

Table 8.3.7 Acid insoluble ash

S.NO	Value(%w/w)
1	2.16
2	2.26
3	2.98
Mean ±S.D	2.46±0.44

Mean ± Standard Deviation (n=3)

Water soluble ash

Table 8.3.8 Water soluble ash

S.NO	Value(%w/w)
1	4.09
2	4.34
3	3.90
Mean ±S.D	4.11±0.22

Mean ± Standard Deviation (n=3)

Sulphated ash

Table 8.3.9 Sulphated ash

S.NO	Value(%w/w)
1	3.5
2	3.9
3	3.3
Mean ± S.D	3.5±0.30

Mean ± Standard Deviation (n=3)

Determination of Water Soluble Extractive value

Water soluble constituents **(**like Alkaloids, Glycosides, Saponins, Phenols, Gums and Carbohydrates) present in the polyherbal formulation were determined. Results were given in Table 8.3.10

Table 8.3.10 Water Soluble Extractive value

S.NO	Value(%w/w)
1	3.6
2	3.9
3	2.8
Mean ±S.D	3.4±0.56

Mean ± Standard Deviation (n=3)

Determination of Alcohol Soluble Extractive value:

The Alcohol soluble components (like Alkaloids, Proteins, Amino acids and Carbohydrates present in the polyherbal formulation were determined as per Pharmacopoeial procedure and in-house specifications; results were given in Table

Table 8.3.11 Alcohol Soluble Extractive value

S.NO	Value(%w/w)
1	1.2
2	1.8
3	1.5
Mean±S.D	1.5±0.3

Mean ± Standard Deviation (n=3)

Crude fibre content

Table8.3.12 crude fibre content

S.NO	Value(%w/w)
1	21.4
2	33.3
3	30.1
Mean ± S.D	28.26±6.1

Mean ± Standard Deviation (n=3)

PHYTOCHEMICAL ANALYSIS

1. PRELIMINARY PHYTOCHEMICAL SCREENING

Table8.3.13 Preliminary phytochemical screening

S.No	Chemical constituents	Present(+) in capsule powder
1	Carbohydrates	+
2	Alkaloids	+
3	Steroids	+
4	Glycosides	+
5	Flavanoids	+
6	Tannins	+
7	Phenolic compounds	+
8	Proteins	+
9	Terpenoids	+
10	Fats & oils	+
11	Mucilage and gums	+

The results were established a scientific data which can be used for the identification of the crude drugs.

FLUORESCENCE ANALYSIS

Table 8.3.14 Fluorescence analysis of polyherbal capsules

S.No	Treatment	Short UV	Ordinary light	Long UV
1	Powder	Light green	Light brown	Colorless
2	Powder + water	Yellowish green	Brown	Brown
3	Powder +Iodine	Black	Black	Black
4	Powder +Picric acid	Yellow	Light brown	Yellowish green
5	Powder + NaOH	Green	Dark brown	Dark brown
6	Powder +Ethanol	Light yellow	Blackish brown	Dark yellow
7	Powder +KOH	Light green	Brown	Black
8	Powder +$FeCl_3$	Black	Light brown	Black
9	Powder +Acetic acid	Light yellow	Light brown	Yellow
10	Powder +Acetone	Green	Brown	Black
11	Powder +Ammonia	Yellowish green	Black	Black
12	Powder +HCl	Yellowish green	Light brown	Dark brown
13	Powder +H_2SO_4	Yellowish green	Light brown	Dark brown
14	Powder +HNO_3	Green	Light brown	Dark brown

The results were established a scientific data which can be used for the identification of the crude drugs.

QUANTITATIVE ESTIMATION

The Polyherbal formulation was found to contain various phytochemical constituents and hence it is desirable to quantify few of them in order to establish a standard to maintain its quality. Among them the estimation of total Alkaloids, Saponins, Flavanoids, and Tannin content in the aqueous extract were decided to be taken as parameters. Samples were drawn from three random samples of polyherbal capsules and the total alkaloids, Saponins, Flavonoids, and Tannin content present in them were estimated. The estimated amounts of Alkaloids, Saponins, Flavanoids, and Tannins were enumerated in the Table8.3.15

Table 8.3.15 Quantitative estimation of phytoconstituent

S.No	Parameters	Values(%w/w)
1	Total Alkaloids	5.44±0.29
2	Total Saponins	7.77±0.59
3	Total Tannins	9.96±0.78
4	Total Flavonoids	3.66±0.36

From the results obtained it is determined that the average content of Alkaloids, Saponins, Flavonoids and Tannins were present in the Polyherbal formulation.

CHROMATOGRAPHIC FINGER PRINTING ANALYSIS

HPTLC CHROMATOGRAM FOR JW 961-01 MMC

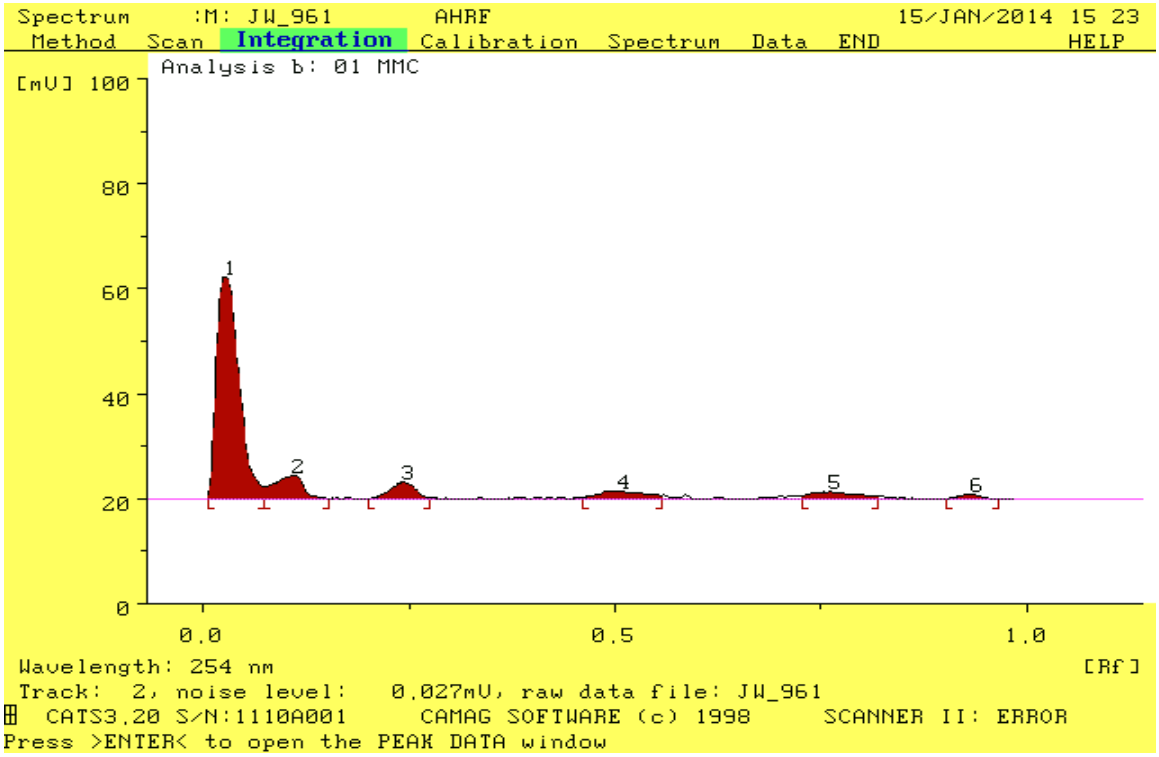


Fig. 8.3.1 Chromatogrphic finger printing analysis

Result table

Table 8.3.16 Rf value of the capsules (Chromatogrphic finger printing)

S. No	Rf	Height	Area	Lambda max(nm)
1	0.02	42.4	878.1	222
2	0.10	4.6	125.6	203
3	0.24	3.2	76.3	205
4	0.50	1.6	67.0	237
5	0.76	1.4	59.6	400
6	0.93	0.9	18.6	200

Fig. 8.3.1 Chromatogrphic finger printing analysis

DETERMINATION OF PESTICIDAL RESIDUE BY TLC

The Polyherbal formulation was tested for the presence of any pesticidal residues by thin layer chromatography using reference standards. The results obtained from the analysis were given in Table 8.3.17

Table8.3.17 Determination of Pesticidal Residues

S.No	Pesticide groups	Pesticides	
		Sample I	Sample II
1	organophosphorus	ND	ND
2	carbamates	ND	ND

ND –No spots were detected

From the results it can be concluded that the formulations were safer as there is no traceable limit of pesticides present in them.

HEAVY METALS ANALYSIS

Capsules were analyzed for the heavy metals which include Arsenic, Lead and Mercury. The results are as follows:

Table 8.3.18 Heavy metals analysis

S.No	Parameters	Results	Limits as per WHO
1	Arsenic	0.05 ppm	MNT5ppm
2	Lead	0.3 ppm	NMT10ppm
3	Mercury	0.001 ppm	NMT0.5ppm

From the results it is shown that the Polyherbal formulation complies with the heavy metals limits of the WHO guidelines and hence it is safe to be taken internally.

MICROBIAL LOAD ANALYSIS:

Microbial screening is done for the Polyherbal formulation from the fresh packs of the Same Batch and the results obtained were detailed in Table8.3.19.

Table 8.3.19 Microbial load analysis

S.No	Parameters	Results	Limits as per WHO
1	Total Microbial Count	110 cfu/g	NMT 1000 cfu/g
2	Yeast and Mould	Nil	NMT 100 cfu/g
3	*E.Coli*	Absent	Should be absent
4	*Salmonella*	Absent	Should be absent
5	*Shigella*	Absent	Should be absent
6	*Streptococcus*	Absent	Should be absent

(Note: *cfu – Colony forming units*)

From the results, it is shown that the formulation complies with the WHO standards for Microbial load analysis and hence it is safer to be taken internally.

8.4 ACCELERATED STABILITY STUDIES

Table 8.4.1 Organoleptic parameters of capsules during the stability period

Parameters	Initial study	30 days	60 days	90 days
Nature	Powder	NC	NC	NC
Color	Brown	NC	NC	NC
Odour	Slight aromatic	NC	NC	NC
Taste	charetristic	NC	NC	NC

NC-No Change

Table 8.4.2 Quality control test

Parameters	**Stability period**		
	After 1 month	**After 2 month**	**After 3 month**
Uniformity of weight	511.8±2.88mg	515.4±1.34mg	515.6±0.56mg
Loss on drying	3.1± 0.26%w/w	3.8±0.12%w/w	3.5±0.06%w/w
Disintegration test	9'24"±0.25	8'56"±0.34	8'14"±0.65
P^H	5.5±0.49	5.7±0.28	5.8±0.43
Total ash	5.1±0.31%w/w	5.3±0.59%w/w	5.5±0.05%w/w
Acid insoluble ash	6.3±0.02%w/w	6.0±0.16%w/w	6.5±0.56%w/w
Water soluble ash	4.6±0.05%w/w	5.2±0.03%w/w	5.3±0.91%w/w
Sulphated ash	3.2±0.26%w/w	3.9±0.65%w/w	3.5±0.03%w/w
Water soluble extractive value	2.8±0.30%w/w	3.0±0.97%w/w	3.1±1.24%w/w
Alcohol soluble extractive	1.53±0.35%w/w	1.59±0.56%w/w	1.56±0.47%w/w
Crude fibre content	30.1±0.35%w/w	31.34±0.34%w/w	31.29±0.74%w/w
Total alkaloid content	5.43±0.43%w/w	5.98±0.85%w/w	5.34±0.54%w/w
Total saponin content	7.39±0.05%w/w	7.90±0.45%w/w	7.91±0.86%w/w
Total tannin content	9.45±0.45%w/w	9.13±0.18%w/w	9.78±0.83%w/w
Total flavonoid content	3.85±0.02%w/w	3.46±0.62%w/w	3.45±0.32%w/w

The values are expressed as mean ± SD. N=3

MICROBIAL LOAD ANALYSIS

Table 8.4.3 Microbial load analysis

S.No	parameters	Results			specification
		Initial study	After 1 month	After 3 month	
1	Total Microbial Count	110cfu/g	110cfu/g	120cfu/g	NMT 1000cfu/g
2	Yeast and Mould	Nil	Nil	Nil	NMT 100cfu/g
3	E.Coli	absent	absent	absent	Should be absent
4	Salmonella	absent	absent	absent	Should be absent
5	Shigella	absent	absent	absent	Should be absent
6	Streptococcus	absent	absent	absent	Should be absent

All the data shows that the formulation is stable under accelerated stability conditions

8.5 PHARMACOLOGICAL STUDIES

***In-vitro* anti arthritic activity**

Table 8.5.1 *In-vitro* anti arthritic activity of extract of ***Asparagus racemosus***

Assay	Percentage inhibition at different concentrations				
	100µg	200µg	400µg	800µg	200µg(std)
Protein denaturation	60.83±0.30	76.52±0.05	80.33±0.42	86.27±0.83	87.12±0.51
Inhibition of protenase enzyme	67.10±0.26	72.91±0.41	76.42±0.18	80.52±0.19	81.53±0.17
Membrane stabilization	59.29±0.16	65.38±0.71	74.84±0.73	81.89±0.35	86.13±1.34

The values are expressed as mean ± SD. N=3.

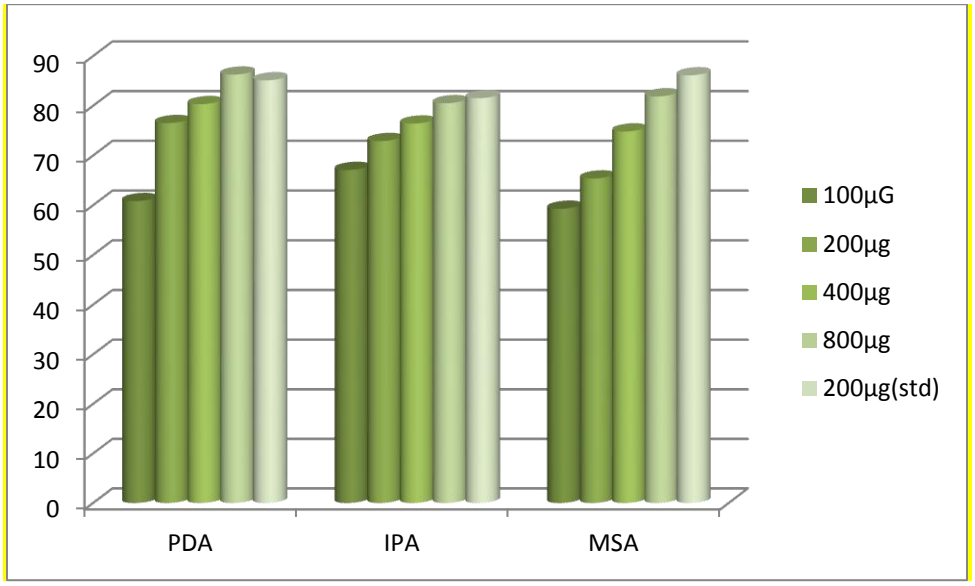


Fig 8.5.1 Graphical representation of in vitro anti arthritic activity extract of ***asparagus racemosus***

PDA- Protein denaturation assay

IPA- Inhibition of protenase enzyme activity

MSA- Membrane stabilization assay

The extract of *Asparagus racemosus* showed a dose dependent inhibitory activity on protein denaturation, protenase enzyme and membrane lysis. The protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (86.27%,80.52%,81.89%) which was less than the standard drug, diclofenac sodium 200 µg(87.12%,81.53%,86.13%).

Table 8.5.2 *In-vitro* anti arthritic activity of extract of ***allium sativum***

Assay	**Percentage inhibition at different concentrations**				
	100µg	**200µg**	**400µg**	**800µg**	**200µg(std)**
Protein denaturation	52.76±1.42	62.39±1.39	72.76±1.15	75.28±1.32	81.32±0.32
Inhibition of protenase enzyme	64.29±0.17	69.36±1.72	74.42±0.09	76.28±0.15	79.49±0.16
Membrane stabilization	55.72±0.64	60.43±0.48	66.32±0.52	78.43±0.26	83.92±0.31

The values are expressed as mean ± SD.n=3

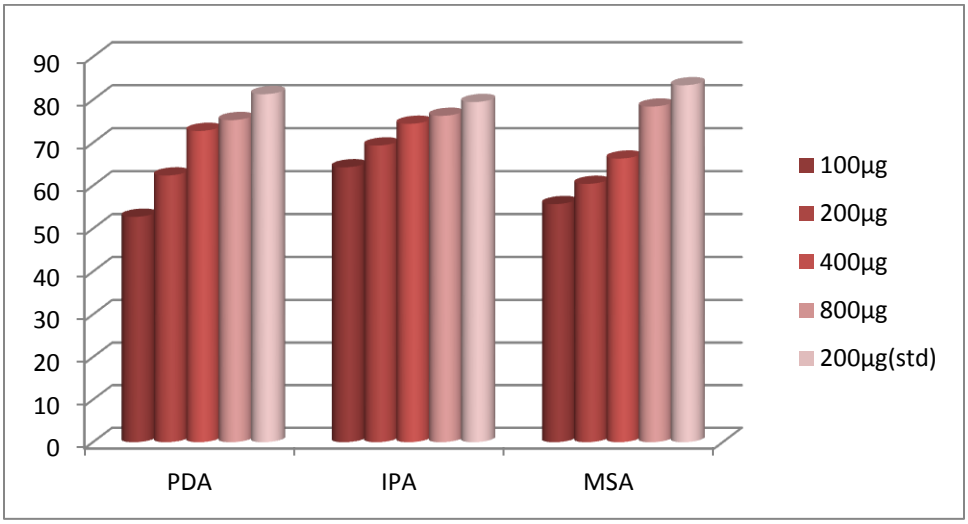


Fig 8.5.2 Graphical representation of invitro anti arthritic extract of ***allium sativum***

The extract of *Allium sativum* showed a dose dependent inhibitory activity on protein denaturation, protenase enzyme and membrane lysis. The protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (75.28%, 76.28%, 78.43%) which was less than the standard drug, diclofenac sodium 200 µg(81.32%,79.49%,83.92%).

Table 8.5.3 *In-vitro* anti arthritic activity of extract of ***Bacopa monneri***

Assay	**Percentage inhibition at different concentrations**				
	100µg	**200µg**	**400µg**	**800µg**	**200µg(std)**
Protein denaturation	60.32±0.25	72.84±1.39	79.43±1.15	84.32±0.95	85.43±0.49
Inhibition of protenase enzyme	56.29±0.38	60.37±0.19	65.39±0.95	74.32±0.15	78.48±1.15
Membrane stabilization	63.21±1.43	70.29±0.48	74.39±0.15	79.53±0.48	81.43±0.86

The values are expressed as mean ± SD. N=3

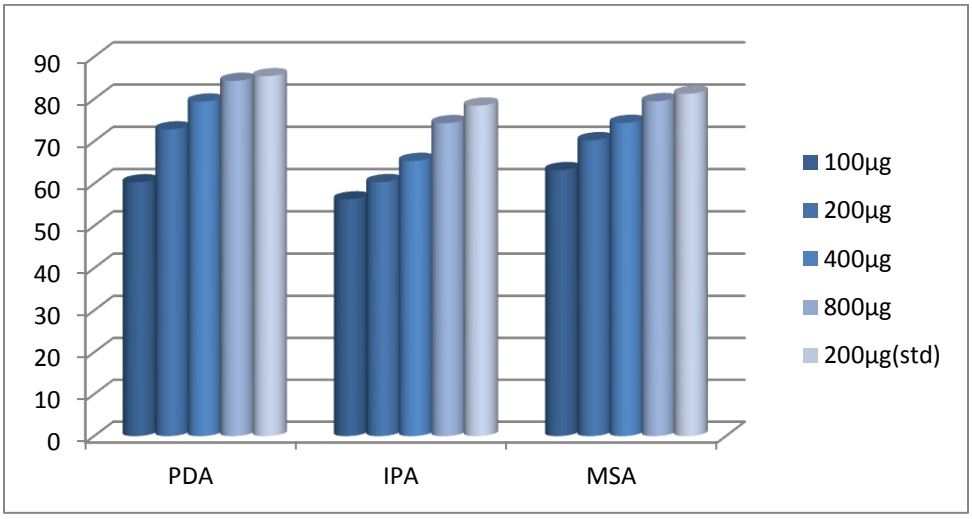


Fig8.5.3 Graphical representation of in vitro anti arthritic extract of ***Bacopa monneri***

The extract of *Bacopa monneri* showed a dose dependent inhibitory activity on protein denaturation, protenase enzyme and membrane lysis. The protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (84.32%, 74.32%, 79.53%) which was less than the standard drug, diclofenac sodium 200 µg(85.43%,78.48%,81.43%).

Table 8.5.4 *In-vitro* anti arthritic activity of extract of ***Lippia nodiflora***

Assay	**Percentage inhibition at different concentrations**				
	100µg	**200µg**	**400µg**	**800µg**	**200µg(std)**
Protein denaturation	74.73±0.32	79.93±0.31	81.73±0.37	85.93±0.73	86.32±0.48
Inhibition of protenase enzyme	69.38±0.38	74.31±0.65	77.83±0.28	79.53±0.65	83.65±0.41
Membrane stabilization	57.38±0.48	61.26±0.29	66.73±0.37	71.42±0.72	74.29±1.13

The values are expressed as mean ± SD. N=3

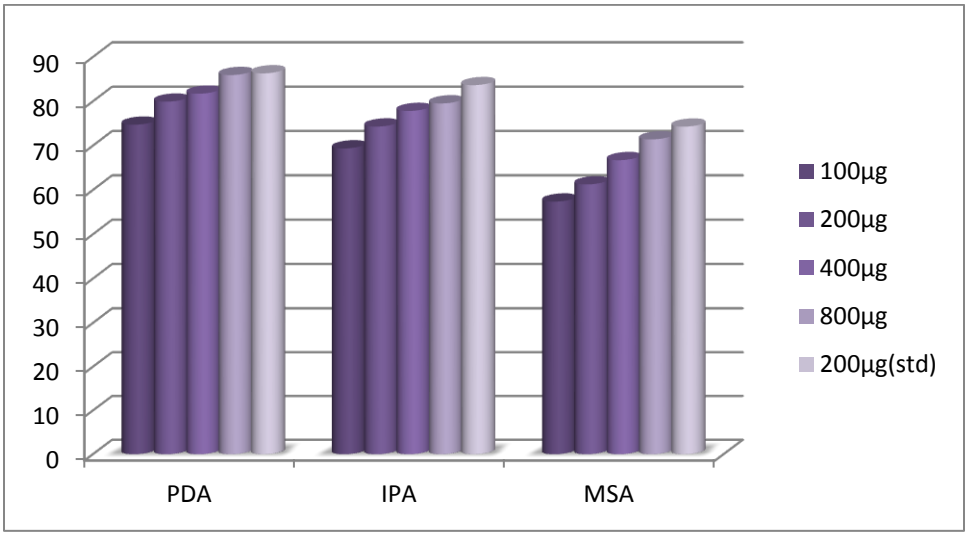


Fig 8.5.4Graphical representation of in vitro anti arthritic extract of ***Lippia nodiflora***

The extract of *Lippia nodiflora* showed a dose dependent inhibitory activity on protein denaturation, protenase enzyme and membrane lysis. The protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (85.93%, 79.53%, 71.42%) which was less than the standard drug, diclofenac sodium 200 µg(86.32%,83.65%,74.29%).

Table8.5.5 in-vitro anti arthritic activity of extract of ***Oldenlandia heynei***

Assay	**Percentage inhibition at different concentrations**				
	100µg	**200µg**	**400µg**	**800µg**	**200µg(std)**
Protein denaturation	70.36±0.16	75.38±0.32	79.49±0.39	82.48±0.47	85.93±0.05
Inhibition of protenase enzyme	68.43±0.39	72.48±0.54	76.48±0.65	80.48±0.82	81.38±0.18
Membrane stabilization	61.38±0.02	67.38±0.29	70.37±0.03	72.48±0.28	74.29±0.19

The values are expressed as mean ± SD. N=3

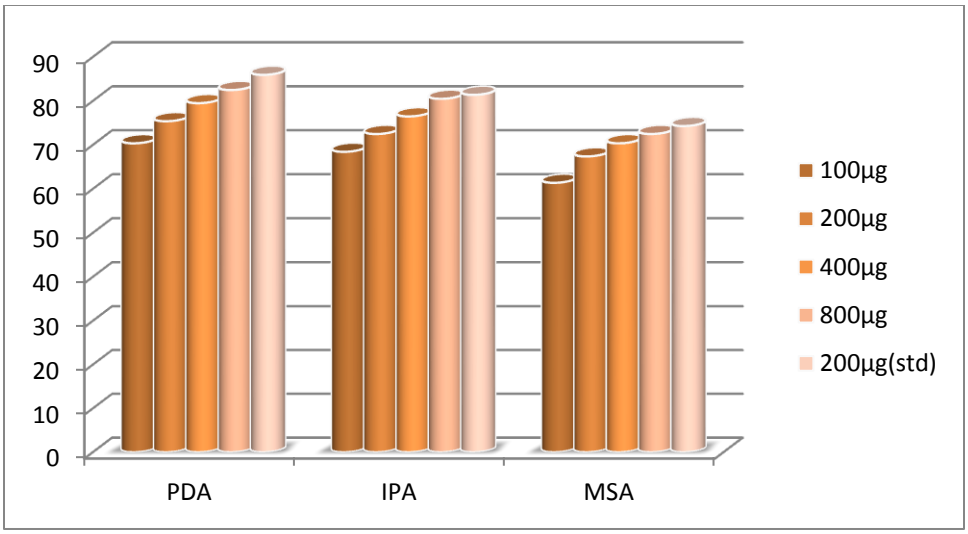


Fig 8.5.5 Graphical representation of in vitro anti arthritic extract of *Oldenlandia heynei*

The extract of *Oldenlandia heynei* showed a dose dependent inhibitory activity on protein denaturation, protenase enzyme and membrane lysis. The protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (82.48%, 80.48%, 72.48%) which was less than the standard drug, diclofenac sodium 200 µg(85.93%,81.38%,74.29%).

Table 8.5.6 in-vitro anti arthritic activity of extract of ***Smilax zeylanica***

Assay	**Percentage inhibition at different concentrations**				
	100µg	**200µg**	**400µg**	**800µg**	**200µg(std)**
Protein denaturation	70.35±0.35	72.38±0.32	75.29±0.17	82.43±0.04	82.57±0.02
Inhibition of protenase enzyme	70.41±0.37	75.37±0.04	80.38±0.26	86.73±0.17	87.36±0.37
Membrane stabilization	64.28±0.93	69.36±0.07	73.72±0.64	75.38±0.08	81.39±1.15

The values are expressed as mean ± SD. N=3

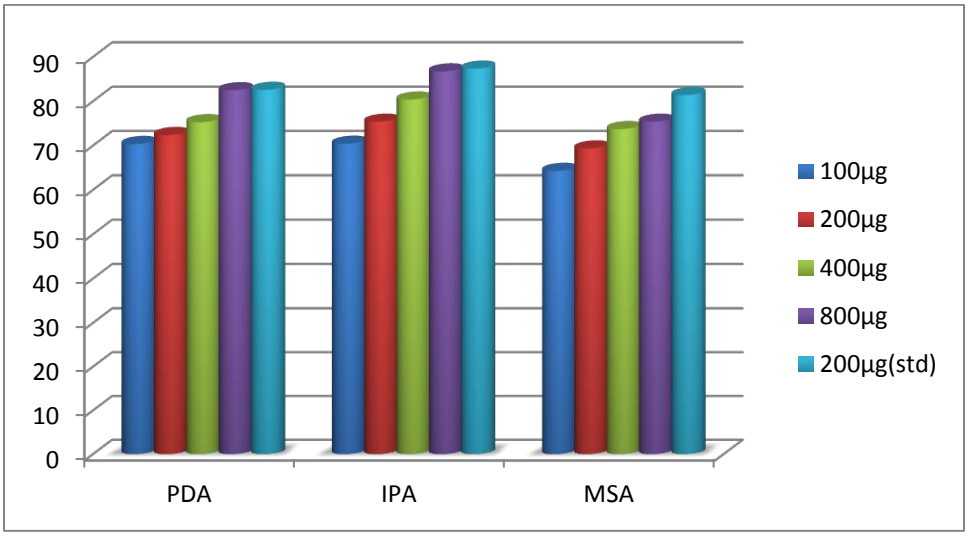


Fig 8.5.6 Graphical representation of in vitro anti arthritic extract of *Smilax zeylanica*

The extract of *Smilax zeylanica* showed a dose dependent inhibitory activity on protein denaturation, protenase enzyme and membrane lysis. The protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (82.43%, 86.73%, 75.38%) which was less than the standard drug, diclofenac sodium 200 µg(82.57%,87.36%,81.39%)

Table 8.5.7*In vitro* antioxidant and anti arthritic activity of polyherbal capsule formulation

Assay	Percentage inhibition at different concentrations				
	100µg	**200µg**	**400µg**	**800µg**	**200µg(std)**
Protein denaturation	45.32±0.42	51.23±0.12	65.84±0.84	74.92±0.43	78.34±0.56
Inhibition of protenase enzyme	54.23±0.22	59.21±0.45	69.43±0.67	76.43±0.47	80.32±0.34
Membrane stabilization	43.23±0.49	49.39±0.34	58.30±0,40	68.32±0.11	72.49±0.19
Reducing power assay	42.48±0.05	52.24±0.84	65.43±0.43	71.53±0.32	72.43±0.56
Nitric oxide scavenging assay	40.53±0.47	51.38±0.48	69..85±0.36	74.27±1.38	75.94±0.75

The values are expressed as mean ± SD. N=3

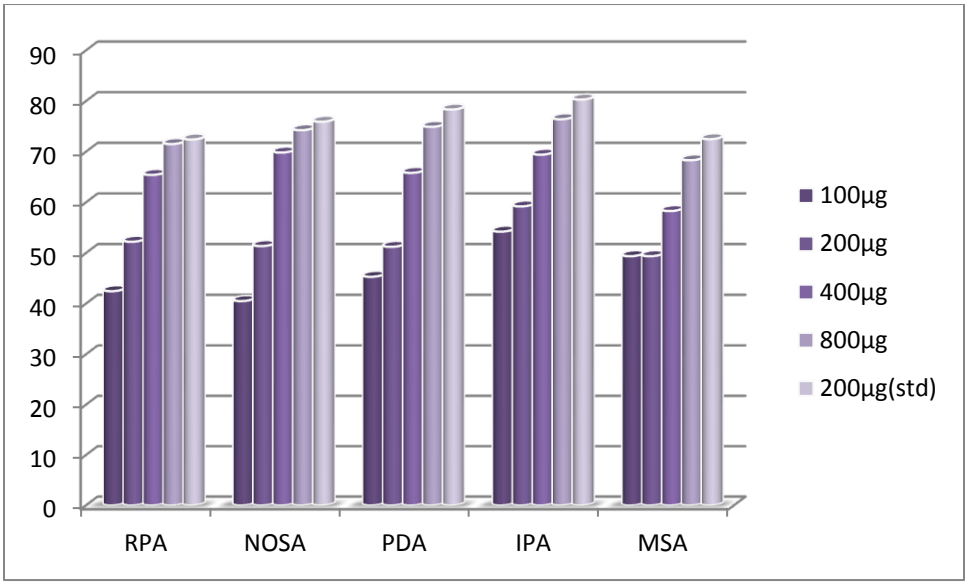


Fig 8.5.7: Graphical representation of anti oxidant and anti arthritic activity of poly herbal formulation of the capsules

The antioxidant activity of capsules showed a dose dependent inhibitory of reducing power assay and nitric oxide scavenging assay. The reducing power assay and nitric oxide scavenging assay was maximum at the concentration 800µg (71.53%, 74.27%,) which was less than the standard drug, ascorbic acid 200 µg(72.43%,75.94%)

8.6ACUTE TOXICITY STUDY

Table 8.6.1 Behavioural and physical observation of polyherbal formulation treated rats (2000mg/kg body weight)

OBSERVATION	**30 mins**	**4 hrs**	**24 hrs**	**14th day**
Body weight	No change	No change	No change	No change
Preterminal deaths	Absent	Absent	Absent	Absent
Cage side observation	Normal	Normal	Normal	Normal
Motor activity	Normal	Normal	Normal	Normal
Convulsions	Absent	Absent	Absent	Absent
Piloerection	Absent	Absent	Absent	Absent
Righting reflex	Present	Present	Present	Present
Lacrimation	Normal	Normal	Normal	Normal
Salivation	Normal	Normal	Normal	Normal
Respiration	Normal	Normal	Normal	Normal
Skin color	Normal	Normal	Normal	Normal
Diarrhoea	Absent	Absent	Absent	Absent
Loss of corneal reflex	Normal	Normal	Normal	Normal
Loss of pinnal reflex	Normal	Normal	Normal	Normal
Grooming	Absent	Absent	Absent	Absent
Sedation	Normal	Normal	Normal	Normal
Excitation	Normal	Normal	Normal	Normal
Aggression	Normal	Normal	Normal	Normal

The results of acute toxicity study are shown in table .There were no morbidity and mortality observed for polyherbal formulation treated animals upto 2000 mg/kg.

8.7 INVIVO ANTIARTHRITIC ACTIVITY

Table8.7.1 Changes in paw swelling (paw circumferences in mm)

Treatment	Group I	Group II	Group III	Group IV	Group V
0^{th} day	0.32±0.015	0.33±0.020	$0.45±0.02^{ab}$	$0.51±0.03^{ab}$	$0.52±0.026^{ab}$
4^{th} day	$0.30±0.004^{b}$	$0.49±0.014^{a}$	$0.35±0.02^{b}$	$0.45±0.021^{a}$	$0.45±0.03^{a}$
8^{th} day	$0.31±0.006^{b}$	$0.48±0.017^{a}$	$0.28±0.03^{b}$	$0.37±0.033^{b}$	$0.37±0.027^{b1}$
14^{th} day	$0.30±0.001^{b}$	$0.52±0.015^{a}$	$0.23±0.02^{b}$	$0.30±0.03^{b}$	$0.23±0.02^{b}$
21th day	$0.32±0.01^{b}$	$0.53±0.014^{a}$	$0.17±0.02^{ab}$	$0.21±0.029^{a1b}$	$0.19±0.041^{ab}$

The values are expressed as mean ±SD, (n=6).

[a] P<0.01 as compared to control ; [a1]P<0.05 as compared to control

[b] P<0.01 as compare to arthritic control ; [b1]P<0.05 as compare to arthritic control

The data was analyzed using one way analysis of variance (ANOVA) followed by Dennett's test. P values <0.01 were considered as Significant.

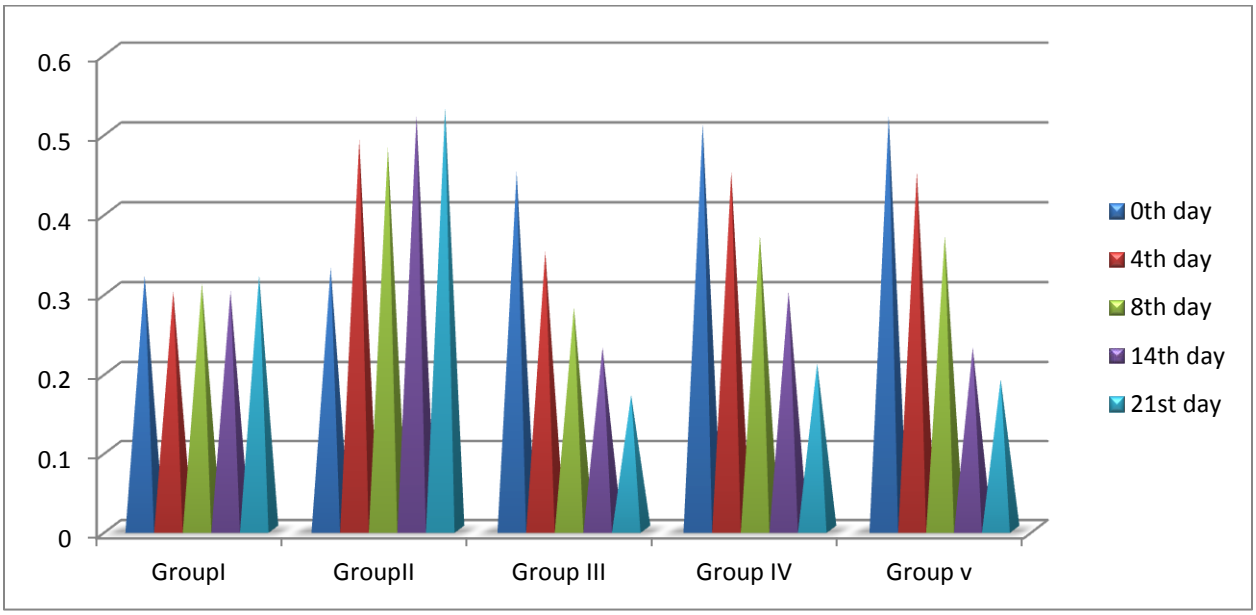


Fig 8.7.1 The graphical representation of paw swelling changes in study groups

Table 8.7.2 Changes in body weight(g)

Treatment	Group I	Group II	Group III	Group IV	Group V
0th day	169.17±4.42[b]	134.14±4.92[a]	137.50±4.45[b]	150.83±2.58	121.67±6.68[b]
4th day	171.67±4.45	125.00±4.59[a]	145.83±2.54	158.67±1.28[b]	124.33±4.65[a]
8th day	171.33±4.42[b]	122.50±4.82[a]	148.33±2.27	162.17±0.90[b]	128.33±4.25[a]
14th day	174.00±4.76[b]	120.0±5.34[a]	151.5±2.22[b1]	165.0±0.89[ab]	133.5±4.00[a]
21th day	175.17±4.06[b]	115.83±5.65[a]	153.50±2.06[b]	168.33±0.65[b]	137.00±3.63[a1]

The values are expressed as mean ±SD, (n=6).
[a] P<0.01 as compared to control ; [a1]P<0.05 as compared to control
[b] P<0.01 as compare to arthritic control ; [b1]P<0.05 as compare to arthritic control
The data was analyzed using one way analysis of variance (ANOVA) followed by Dennett's test. P values <0.01 were considered as Significant.

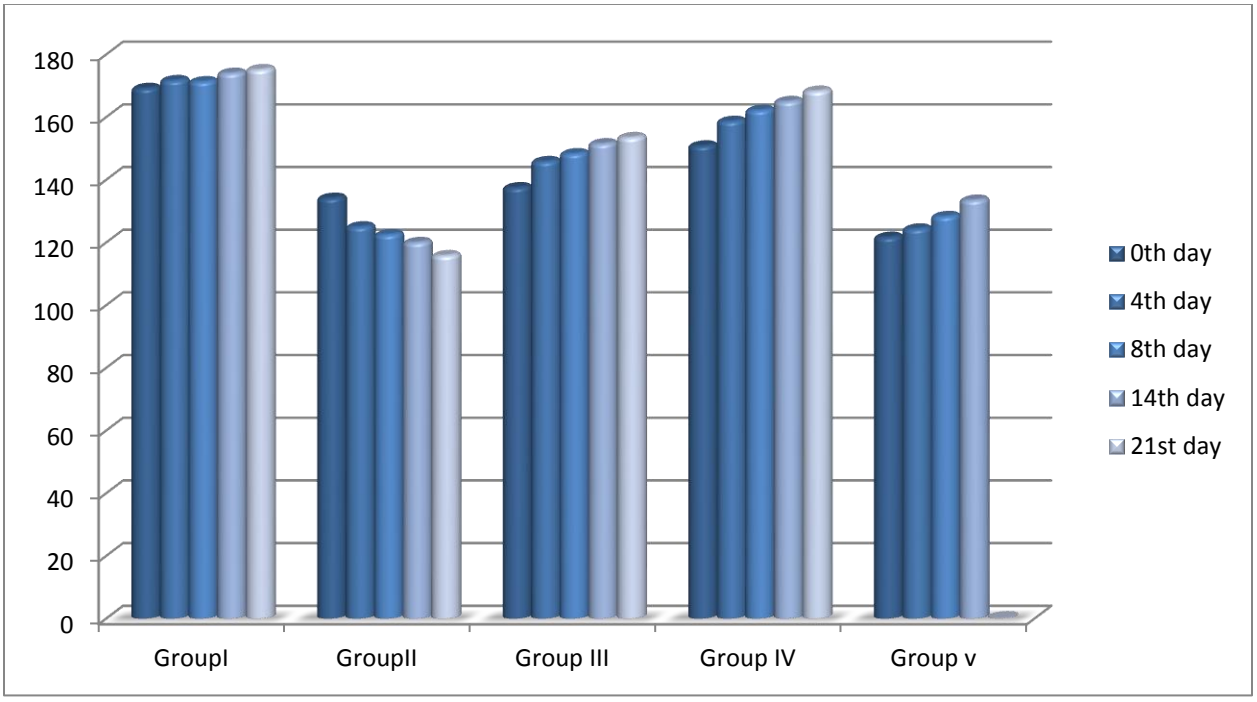


Fig 8.7.2 The graphical representation of body weight changes in study groups

Table 8.7.3 Percent inhibition of paw swelling

Treatment	4th day	8th day	14th day	21st day
Group III	35.24±0.15	39.55±2.23	65.32±0.21	72.32±1.23
Group IV	24.56±1.32	29.32±1.42	48.13±1.42	54.75±2.53
Group V	30.45±2.90	41.23±1.03	50.32±1.32	69.64±1.17

The values are expressed as mean ±SD, (n=6).

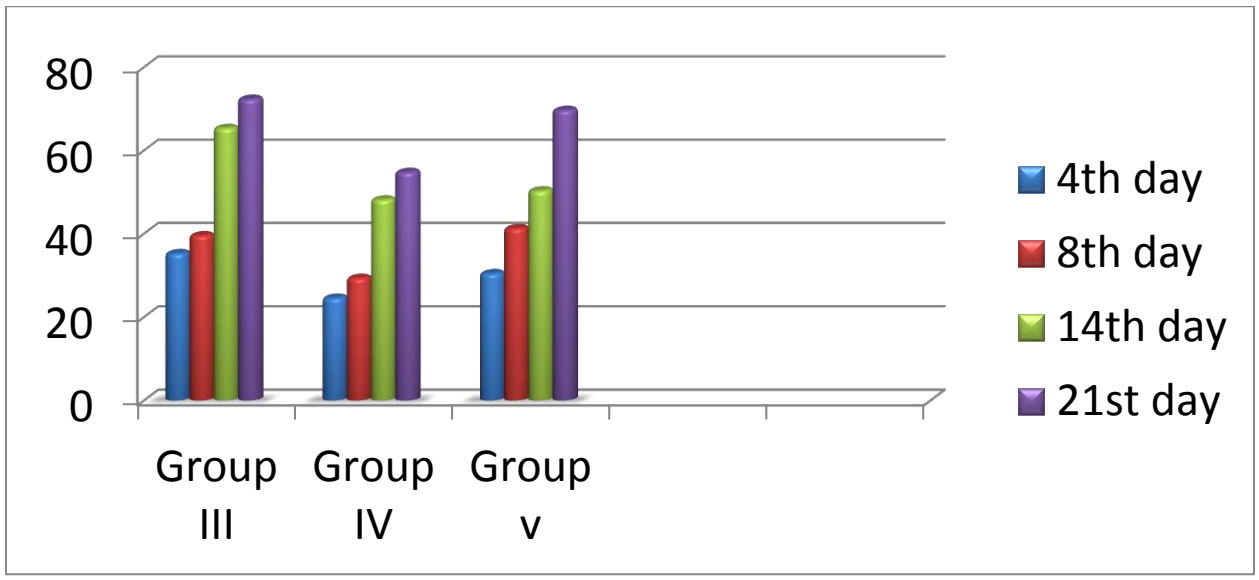


Fig 8.7.3 The graphical representation of percentage inhibition of paw swelling

8.8 HEMATOLOGICAL PARAMETERS

Table8.8.1 Hematological parameters

parameters	**Group I**	**Group II**	**Group III**	**Group IV**	**Group IV**
WBC(Cells/cumm)	6.5±0.3	7.4± 0.41	7.1±0.32	6.9±0.83	6.4±0.2
RBC(Cells/cumm)	6.48±0.21	5.83±0.13	6.14±0.22	6.03±0.63	6.76±0.02
DC-lymphocytes	24.8±0,42	50.2±0.42	36.9±0.38	35.0±0.45	30.49±0.05
DC- monocytes	4.12±0.14	3.26±0.32	5.32±0.87	3.65±0.39	4.83±0.01
DC- eosinophils	1.34±0.12	2.45±0.74	1.55±0.34	1.33±0.08	1.39±0.17
DC- neutrophils	71.34±0.14	46.12±0.43	57.32±12	61.34±0.41	65.21±0.18
PCV(%)	33.2±0.32	24.8±0.04	32.3±0.54	33.16±0.64	36.93±0.74
ESR(mm/hrs) 30 min	2.32±0.02	1.3±0.32	1.1±0.21	3.02±0.07	2.12±0.77
60 mins	5.12±0.28	21.9±1.21	6.42±0.42	4.5±0.35	5.76±0.09
Hb(g/dl)	11.3±0.45	9.4±0.61	10.35	10.0±0.07	11.9±0.57

The values are expressed as mean ±SD, (n=6).

Hematological parameters did not shows any difference between the polyherbal formulation treated groups, diclofenac sodium treated group and normal control. The arthritic control group there was elevated in Erythrocyte Sedimentation Rate and Reduce Haemoglobin, Red Blood cell, Differential Count on comparing with normal control. This shows that the polyherbal formulation does not affect the hematological parameters.

8.9 RADIOGRAPHIC STUDY

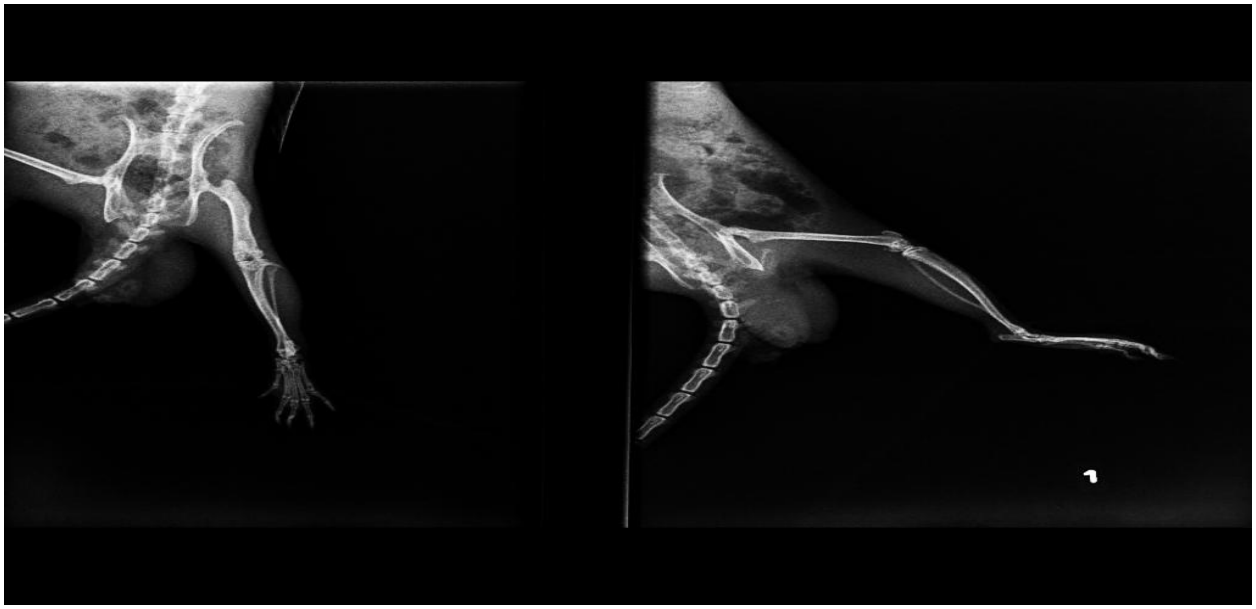


Fig8.9.1 Radiographic hind leg image of control group rat

Fig8.9.2 Radiographic hind leg image of arthritic control group rat

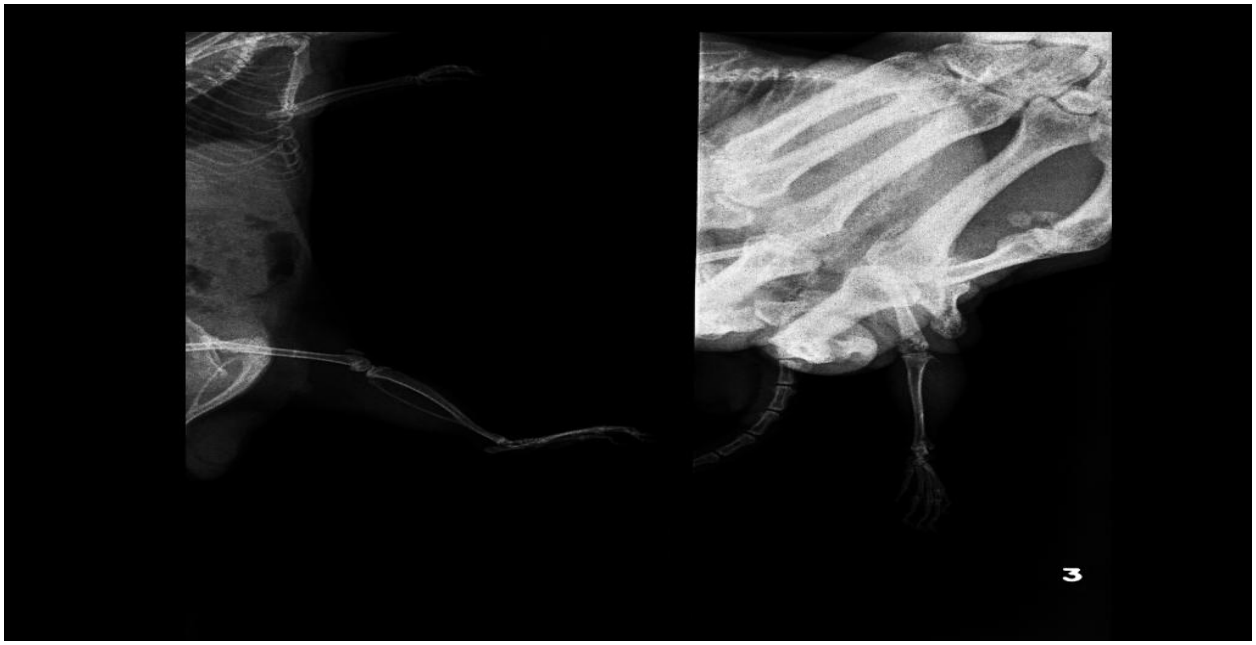


Fig 8.9.3 Radiographic hind leg image of Diclofenac sodium (std) treated group rat

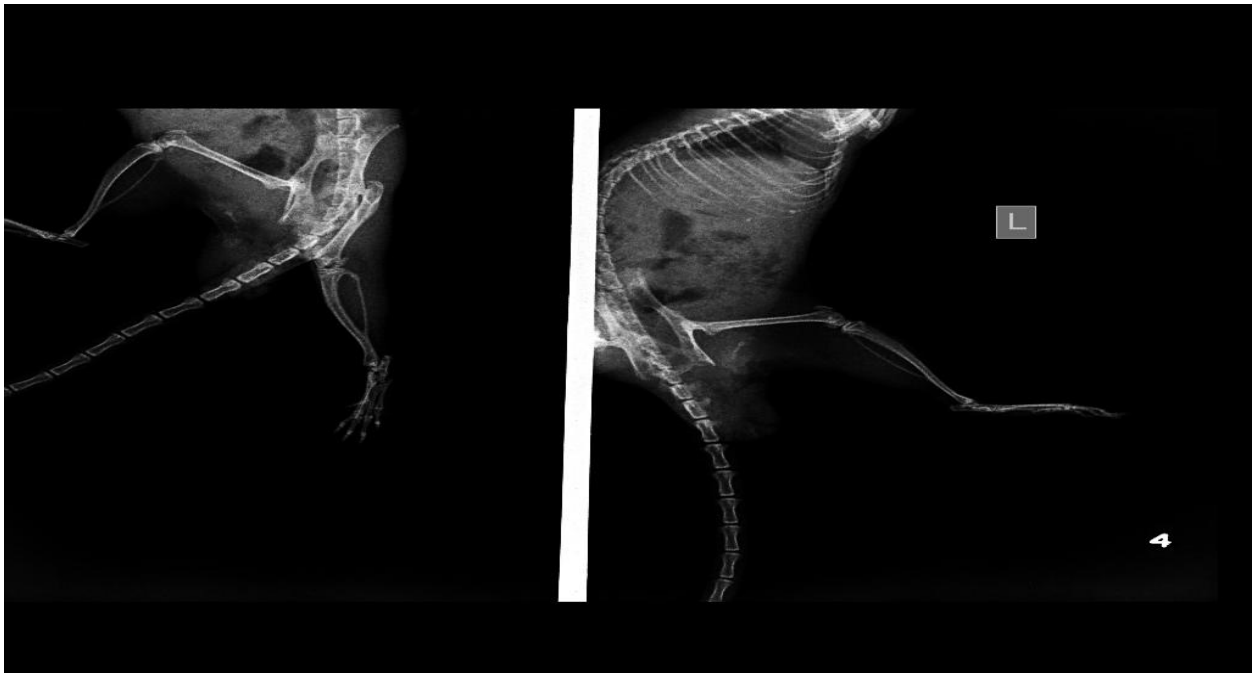


Fig 8.9.4 Radiographic hind leg image of Test drug I treated group rat

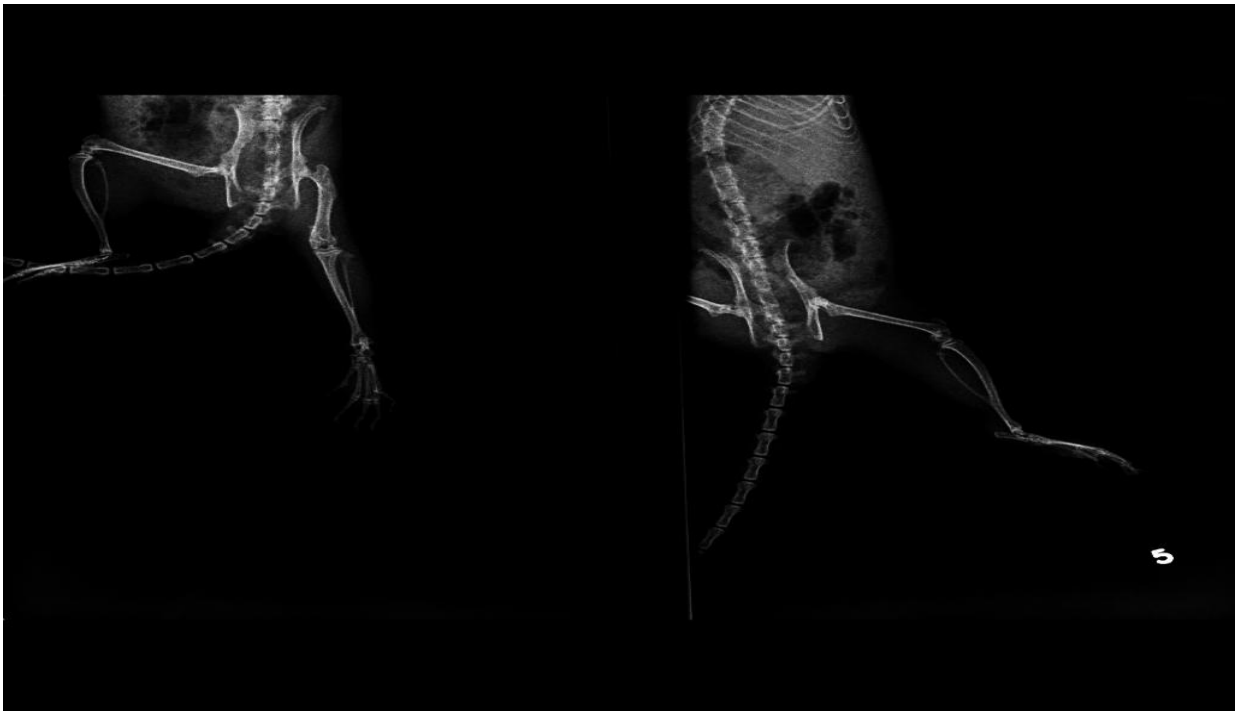


Fig 8.9.5 Radiographic hind leg image of test drug II treated group rat

Report:

Control: No lesion

Arthritic control:

Severe arthritis changes noticed in ankle joint and space lesion also noticed.

Standard:

Mild arthritis changes seen in ankle joint.

Test drug I and II:

Radiographically no abnormalities of knee joint ankle joint seen both test drug treated group.

8.10 HISTOPATHOLOGICAL EXAMINATION OF BONE:

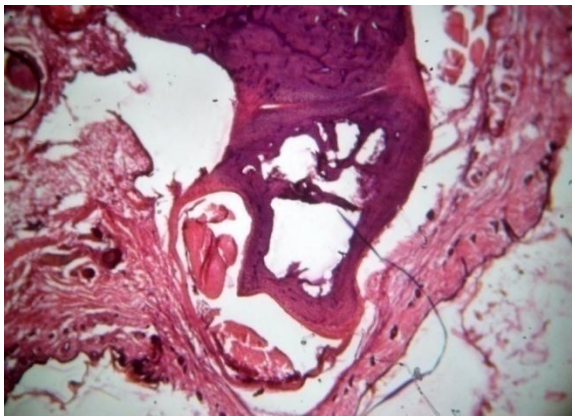


Fig 8.10.1 Examination of bone of control group

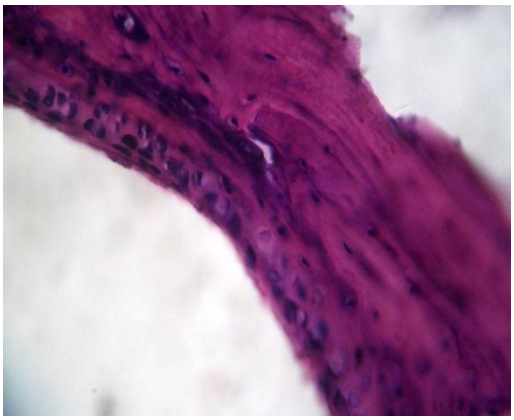


Fig 8.10.2 Examination of bone of arthritic control group

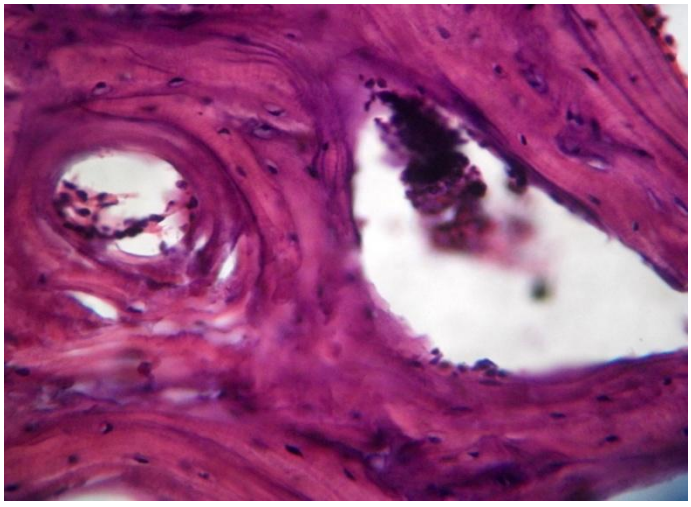


Fig 8.10.3 Examination of bone of diclofenac sodium treated group

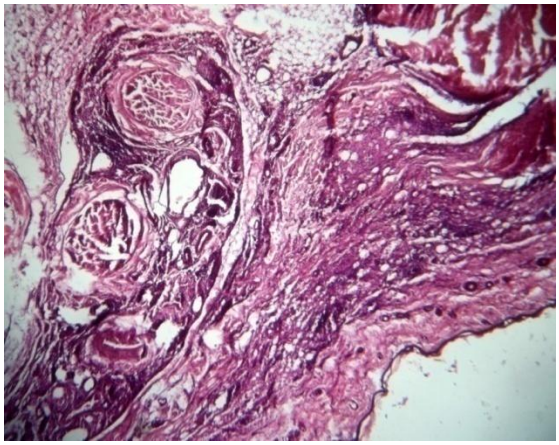


Fig 8.10.4Examination of bone of test drug I

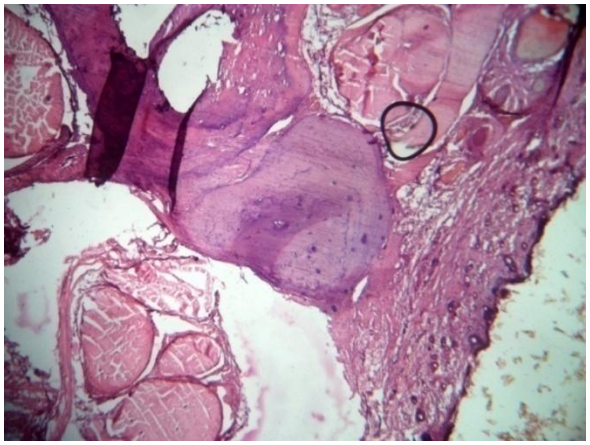


Fig 8.10.5 Examination of bone of test drug II

Result:

Control:

No abnormality detected

Arthritic control:

Severe erosion of cartilage

Standard :

No abnormality detected

Test drug I:

Mild erosion of cartilage

Test drug II

Mild synovial hyperplasia

9. SUMMARY AND CONCLUSION

The present study was attempt to development, standardization and pharmacological activity of anti arthritic polyherbal formulation.

Based on the extensive review of literature, six active ingredients were selected for the formulation of polyherbal capsules to treat rheumatoid arthritis.

The raw materials which are procruded from TTK Health care Ltd are subjected to various raw material analysis for their identy, quality and purity. The materials which complied with the specification were taken for further studies

Preformulation studies such as bulk density, tapped density, compressibility index, housner's ratio and angle of repose were done for the all raw materials.

From the preformulation studies best batches was selected to formulate capsules.

The formulated capsules were standardized as per WHO guidelines using various parameters and include quantification of phytoconstituent, HPTLC, Heavy metals. Stability studies of the capsules are carried out as per ICH guidelines.

Pharmacological studies (both *in vitro* and *in vivo*) were carried out to study the efficacy of the formulation in the treatment of rheumatoid arthritis and *in vitro* Anti oxidant activity of formulated capsule also done.

The extract of *asparagus racemosus* showed the protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (86.27%,80.52%,81.89%) which was less than the standard drug, diclofenac sodium 200 µg(87.12%,81.53%,86.13%).

The extract of *allium sativum* showed the protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (75.28%, 76.28%, 78.43%) which was less than the standard drug, diclofenac sodium 200 µg(81.32%,79.49%,83.92%).

The extract of *Bacopa monneri* showed the protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (84.32%, 74.32%, 79.53%) which was less than the standard drug, diclofenac sodium 200 µg(85.43%,78.48%,81.43%).

The extract of *Lippia nodiflora* showed the protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (85.93%, 79.53%, 71.42%) which was less than the standard drug, diclofenac sodium 200 µg(86.32%,83.65%,74.29%).

The extract of *Oldenlandia heynei* showed the protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (82.48%, 80.48%, 72.48%) which was less than the standard drug, diclofenac sodium 200 µg(85.93%,81.38%,74.29%).

The extract of *Smilax zeylanica* showed The protein denaturation, protenase enzyme and membrane stabilization was maximum at the concentration 800µg (82.43%, 86.73%, 75.38%) which was less than the standard drug, diclofenac sodium 200 µg(82.57%,87.36%,81.39%).

The antioxidant activity of capsules showed the reducing power assay and nitric oxide scavenging assay was maximum at the concentration 800µg (71.53%, 74.27%,) which was less than the standard drug, ascorbic acid 200 µg(72.43%,75.94%).

Acute toxicity studies were performed according to OECD guidelines and for *invivo* studies was fixed.

Invivo studies using complete freund's adjuvants induced model was done in rats. The test drug shows results are body weight, paw swelling, hematologic, histopathologic and radiographic study was done.

Body weight of rat increased in polyherbal formulation treated groups and also diclofenac in the diclofenac treated groups. There was a statistically significance increase the body weight, in the polyherbal formulation treated groups on comparing with arthritic control group.

The paw swelling showed a decrease in polyherbal formulation treated groups and also diclofenac sodium treated group. There was a statistically significance decrease the paw swelling in the polyherbal formulation treated groups on comparing with arthritic control group.

Hematological parameters did not shows any difference between the polyherbal formulation treated groups, diclofenac sodium treated group and normal control. The arthritic control group there was elevated in RBC, DC, ESR and Hb on comparing with normal control. This shows that the polyherbal formulation does not affect the hematological parameters.

Histopathologic examination of the joint showed severe cartilage erosion in arthritic control group compared with polyherbal formulation treated group and diclofenac sodium treated group. This shows that polyherbal formulation suppressed the inflammation changes associated with arthritis which was equivalent to diclofenac sodium.

Radiographic study showed mild lesion in polyherbal formulation and standard drug treated group compared to arthritic control group having severe arthritic change. This shows that polyherbal formulation suppressed the inflammation changes associated with arthritis which was equivalent to diclofenac sodium.

Future studies can be directed towards the exact mechanism of action responsible for this anti arthritic activity and further the study can be extended for clinical trials also.

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Binomial: Hedyotis herbacea L.

Family: Rubiaceae

Synonym(s): Oldenlandia herbacea R.Br ex G. Don
O. herbacea (L.) Roxb

Regional names: .....

Reg.No of the certificate: PARC/2013/2165

References: Nair, N.C & Henry, A.N. Flora of TamilNadu, India I: _____ .1983.
Henry, A.N. *et al.* Ibid. II: p. 8 .1987.
Ibid. _____ III: _____ .1989.

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Binomial: Asparagus racemosus Willd.

Family: Liliaceae.

Synonym(s): —

Regional names: Tam: Ammaikodi, Thanneer vittan Kizhangu.

Reg.No of the certificate: PARC/2013/2164.

References: Nair, N.C & Henry, A.N. Flora of TamilNadu, India I: —— .1983.

Henry, A.N. *et al.* Ibid. II: —— .1987.

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Binomial: Bacopa monnieri (L.) Pennell

Family: Scrophulariaceae.

Synonym(s): Lysimachia monnieri L.

Regional names: Tam: Nirbrahmi.

Reg.No of the certificate: PARC/2013/2162.

References: Nair, N.C & Henry, A.N. Flora of TamilNadu, India I: ____ .1983.
Henry, A.N. *et al.* Ibid. II: pg: 119 .1987.
Ibid. III: ____ .1989.

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AUTHENTICATION CERTIFICATE

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Binomial: Phyla nodiflora (L.) Greene

Family: Verbenaceae

Synonym(s): Verbena nodiflora L., Lippia nodiflora (L.) A. Rich.

Regional names: Tam: Podutalai

Reg.No of the certificate: PARC/2013/2163.

References: Nair, N.C & Henry, A.N. Flora of TamilNadu, India I: —— .1983.

Henry, A.N. *et al.* Ibid. II: pg: 166 .1987.

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PLANT ANATOMY RESEARCH CENTRE

Prof.**P Jayaraman, Ph.D**
Director
Retd, Professor, Presidency College Chennai-5

AUTHENTICATION CERTIFICATE

Based upon the Organoleptic /macroscopic /~~microscopic~~ examination of fresh /~~market~~ sample, it is certified that the specimen given by Ms. J. GEETHA, M. Pharm., Dept of Pharmacognosy, Madras Medical College, Chennai is identified as below:

Binomial: *Allium Sativum L.*

Family: Alliaceae

Synonym(s): —

Regional names: Tam. Vellaippoondu

Reg.No of the certificate: PARC/ 2013/ 2167

References: Nair, N.C & Henry, A.N. Flora of TamilNadu, India I:_____ .1983.

Henry, A.N. *et al.* Ibid. _____ II:_____ .1987.

Ibid. III: p. 41 .1989.

Date: 28. 12. 2013.

(Prof.P.JAYARAMAN)

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AUTHENTICATION CERTIFICATE

Based upon the Organoleptic /macroscopic /~~microscopic~~ examination of ~~fresh~~ /market sample, it is certified that the specimen given by Ms. J. Geetha, II M.Pharm, Dept. of Pharmacognosy, Madras Medical College, Chennai, is identified as below:

Binomial: *Smilax zeylanica* L.

Family: Smilacaceae

Synonym(s): Nil

Regional names: Tam. Kattukodi

Reg.No of the certificate: PARC/2013/2166

References: Nair, N.C & Henry, A.N. Flora of TamilNadu, India I:_____.1983.

Henry, A.N. *et al.* Ibid. _____ II: _____.1987.

Ibid. III: p. 42 .1989.

Date: 29.11.2013

(Prof.P.JAYARAMAN)

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CERTIFICATE

This is to certify that Miss.J.GEETHA, Post Graduate student, Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai-03 had submitted her protocol (Part B application) Vide. 2/243/CPCSEA for the dissertation Programme to the Animal Ethical Committee, Madras Medical College, Chennai-03.

TITLE

ANTI ARTHRITIC POLYHERBAL FORMULATION: DEVELOPMENT, STANDARDIZATION AND EVALUATION"

The Animal Ethical Clearance Committee experts screened her proposal No:Vide. 2/243/CPCSEA and have given clearance in the meeting held on 22.11.13 at Dean's Chamber in MMC, Chennai-03. Her study involves only small animals of house dwelling rats.

17.2.14.

Signature

SPECIAL VETERINARY OFFICER
ANIMAL RESEARCH
MADRAS MEDICAL COLLEGE
CHENNAI-600 003

FACULTY OF PHARMACY
SRI RAMACHANDRA UNIVERSITY

(Declared under Section 3 of the UGC Act, 1956)
Porur, Chennai - 600 116.
Accredited by NAAC with 'A' Grade

HERBOTECH "2012"

Certified that Dr. / Mr. / Ms. J. Geetha *has participated as a Delegate / ~~Organiser~~ / ~~Volunteer~~ / ~~Presented the scientific paper (Oral/Poster)~~ in* ***Herbotech "2012"****, A National Conference on Approaches of Nanotechnology in Herbal Drug Development held on 20th - 22nd November 2012 at Sri Ramachandra University, Porur, Chennai-600 116.*

This conference offers 12 credit hours.

D. Chand
DR. D. CHAMUNDEESWARI
Chief Convener

Dr. K. V. SOMASUNDARAM
Dean of Faculties


64th INDIAN PHARMACEUTICAL CONGRESS
Theme: Pharmacy Education:
Innovation, Strategies and Globalization
Certificate of Participation
This is to certify that
Dr./Prof./Mr./Ms. J. GEETHA
participated in the 64th Indian Pharmaceutical Congress held at
SRM University, Chennai, 7th - 9th December 2012
K. CHINNASWAMY
President
S. V. VEERRAMANI
Vice Chairman
J. JAYASEELAN
Organizing Secretary-R
B. G. SHIVANANDA
Organizing Secretary
Host:
Association of Pharmaceutical
Teachers of India (APTI)